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Bone as a Tissue

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BONE AS A TISSUE

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Preface

Bone as a Tissue because of its wide application to many fields of medicine was selected as the subject for discussion at the annual research conference at the Lankenau Hospital October 30–31 1958 This subject was of particular interest to the Lankenau group in connection with its current research program involving many aspects of connective tissue disorders, including arthritis and rheumatic diseases. Furthermore it was considered timely to review the osseus system in view of the recent advances in this field which may have a profound influence upon future avenues of bone research

We are indebted to the speakers and participants in the discussions for their valuable contributions to our basic understanding of bone growth and development and the application of these basic principles to clinical medicine We are particularly grateful to Dr Franklin C McLean for his valuable assistance in arranging the program

Dr Edward L. Bortz played an important role in the initiation of this conference and was instrumental in securing the necessary financial support, for which we are extremely appreciative The financial support from the William S Merrell Company which enabled us to arrange this conference without charging any registration fee to the participants is gratefully acknowledged

The administration of the Lankenau Hospital, members of the Medical and Research staff the Health Education Department, and individuals connected with the hospital all contributed to the solution of the many aspects of the problems involved in arranging this conference We wish to express our sincere gratitude to all these individuals, too numerous to be mentioned individually who helped make this conference possible

The Editorial Committee

The Lankenau Hospital

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Part I

Osteoporosis

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Osteoporosis of Cushing's Syndrome

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Introduction

In 1932, Cushing[1] described a series of eight cases of an interesting syndrome which he termed "pituitary basophilism" Cushing's patients, all young adults showed the following clinical features an unusual type of increase in subcutaneous fat involving the face neck, and trunk osteoporosis of the skeleton most marked in the spine resulting in kyphosis and diminution of stature amenorrhea in females and ultimate impotence in males hirsutism involving the face and trunk in females atrophic skin with purplish striae hypertension diabetes and polycythemia It has since been recognized, particularly as a result of the work of Albright that the syndrome results from the hypersecretion of adrenal corticosteroid hormones it appears that the "glucocorticoid" hormones (the 11-oxy 17 hydroxy corticosteroids which include cortisone and hydrocortisone) are the ones chiefly concerned, although alterations in symptomatology from case to case may result from variation in the hormones secreted The adrenal overactivity can be either primary as with a secreting adrenocortical tumor or the result of a basophil adenoma of the pituitary

The metabolic concept of the disease introduced by Albright is that it consists of an abnormality of protein and carbohydrate metabolism protein synthesis is diminished and conversion of protein to carbohydrate is increased

If the metabolic disturbance continues for a sufficiently long period a severe degree of osteoporosis occurs, as was noted for instance in six of Cushing's original cases This osteoporosis is of interest for a number of reasons not least because of our definite knowledge of the biochemical background responsible for it In this respect it contrasts with the much

more common "idiopathic" osteoporosis of older people, where there is still considerable debate with regard to the biochemical or hormonal abnormality concerned.

There are two aspects of the subject that I propose to discuss today First, I will look at the structural bone changes in Cushing's syndrome and compare them with the effects of cortisone administration in experimental animals Secondly I will consider the more general question of the

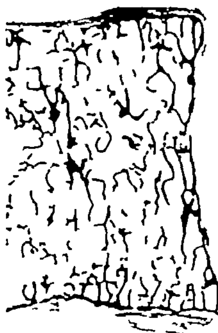


FIG. 1 1 Outline drawing from histologic section of normal lumbar vertebra. (×4) (*Sissons J Bone & Joint Surg* 38B, 418 1956)

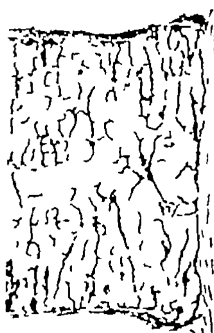


FIG. 1 2. Outline drawing from histologic section of lumbar vertebra in Cushing's syndrome. (×4) (*Sissons J Bone & Joint Surg* 38B, 418 1956)

recognition and assessment of the cellular processes responsible for the maintenance of normal bone structure and for the development of various forms of bony rarefaction.

Bone Changes in Cushing's Syndrome

As already noted, the skeletal abnormality in Cushing's syndrome is an *osteoporosis* i e., a reduction in the amount of bone present without any interference with its mineralization Radiologically (Wang and Robbins,[3] Holland et al [4]) there is a general reduction in bone density this is particularly evident in the spine pelvis, and ribs, although it is present to a less extent in the skull and in the long bones of the limbs. Vertebral bodies

show loss of their trabeculation with relative prominence of their end plates, and the intervertebral discs are expanded or "ballooned" at the expense of the adjacent bone Cortical bone in various situations is reduced in thickness, and fractures of vertebral bodies and of ribs are common

Histologic studies of bone changes in Cushing's syndrome are few[5] but they clearly establish that the thickness of both cortical bone and trabecular structures is reduced sometimes markedly so (see Figs 1 1 to 1-4)



Fig 1 3 An individual bone trabecula from the central part of a normal lumbar vertebral body (×115)



Fig 1-4 An individual bone trabecula from the central part of a severely osteoporotic lumbar vertebral body in Cushing's syndrome. (×115)

In the spine the ballooning of the intervertebral discs and the relative prominence of the end plates of the vertebral bodies are the result of compression of the weakened bone and are accompanied by all degrees of vertebral fracture from damage to isolated trabeculae to completely collapsed bodies.

Cellular Mechanism of Bone Changes. It is common knowledge that a normal skeletal structure results from a balance between two continuous and opposed activities—bone formation and bone destruction. It is clear that a progressive reduction in the amount of bone tissue present might result from increased bone destruction, decreased bone formation, or a combination of the two Histologically we can recognize these two proc

esses which are usually referred to as osteoblastic bone formation and osteoclastic bone destruction.

In Cushing's syndrome, a number of workers (Mooser[6] Rutishauser[7] and Follis[8]) have commented on the scarcity of osteoblasts and of areas of recently formed bone and it is clear that these changes indicate a de crease in osteoblastic activity this is usually regarded as an adequate explanation for the progressive reduction in the amount of skeletal tissue present the same authors failing to observe any increased osteoclasis In 1956[5] I reported on bone changes in four postmortem cases of Cushing's syndrome and made the same comment on the diminution of osteoblastic activity and the absence of any obvious exaggeration of osteoclasis In the course of preparing the present paper I have been able to study bone ma terial from a further 10 cases coming to autopsy at various teaching hospitals in London I have still been unable to find any evidence of exaggerated osteoclasis although I should make the reservation that in all cases the material has been limited to a few sections of vertebral bodies and ribs. A point to which I will return is the need for careful morphologic studies of bone tissue from examples of generalized bone disease including a variety of skeletal sites, and the use of all the modern methods of investigation at our disposal



Fig 1 5 A compression fracture through the upper part of an osteoporotic vertebral body in Cushing's syndrome (×17)

Not only is osteoblastic activity diminished throughout the skeleton in Cushing's syndrome but there is evidence that the bones fail to respond to fracture in a normal manner[5] In Figs. 1 5 and 1-6 for instance, a recent vertebral fracture shows interruption and displacement of bone trabeculae with some local hemorrhage but only slight fibroblastic and osteoblastic cellular activity The not infrequent rib fractures often show persistence of abnormall taining cartilage in the callus[8]

Comparison with the Effects of Experimentally Administered Cortisone

There is a parallel between these findings in Cushing's syndrome and the effects of experimental administration of cortisone in laboratory animals. Since cortisone became available in 1949 a great deal of work has been published concerning its effects on metabolic and cellular processes. Much of this earlier work was reviewed by Ingle and Baker.[9] In sufficiently large doses cortisone inhibits the formation of both ground substance and collagen in a variety of connective tissues and suppresses the inflammatory response to chemical or bacterial agents. Its effect on fracture repair has been studied by numerous workers, including Blunt et al[10] and Sissons and Hadfield.[11] The development of granulation tissue in the fractured region is inhibited (Figs 1 7 1 8) and the expected periosteal new bone (Figs 1 9 1 10) and cartilaginous callus (Figs. 1 11 1 12) fail to develop in normal amounts. Such cartilaginous callus as is ultimately produced fails to show its normal basophilic staining reaction and also fails—at least for a time—to undergo normal replacement by bone.



FIG 1 6 Part of the fractured region from Fig 1 5 Note the relatively inactive osteoblasts (×105)

In young animals longitudinal bone growth is inhibited by cortisone administration[12-14] this result being due to decreased proliferation of the cells of the growing epiphyseal cartilage plates. Children with Cushing's syndrome[15, 16] or those receiving prolonged cortisone therapy[17] show a similar retardation of growth.

The effect of cortisone administration on the structure of preexisting bone tissue does not appear to have received much study in experimental animals, although it is now well known that osteoporosis not infrequently follows prolonged cortisone therapy in human beings. One interesting study however is that of Storey[18-20] who has examined the effects of cortisone administration on the bone of the skull vault and the tooth sockets in rabbits. In these situations bony rarefaction is produced: this is shown by an increased number of resorption cavities, some of which contain osteoclasts. Storey interprets his results as indicating early rapid destruction of bone as well as inhibition of new bone formation to the



Fig. 1 7 Experimental fracture. Control rabbit. Granulation tissue at 4 to 5 days. (×200)



Fig. 1-8 Experimental fracture. Cortisone-treated rabbit. Complete absence of granulation tissue at 4 to 5 days. (×200)



Fig. 1 9 Experimental fracture Control rabbit. Active periosteal bone formation at 4 to 5 days. (×200)



Fig. 1 10 Experimental fracture. Cortisone-treated rabbit. Complete absence of periosteal bone formation at 4 to 5 days. (×200)



FIG. 1 11 Experimental fracture. Control rabbit. Abundant formation of cartilage in callus tissue at 4 to 5 days. (×200)



FIG 1 12 Experimental fracture. Cortisone-treated rabbit. Sparse formation of cartilage in callus tissue at 4 to 5 days. (×200)

present writer however it seems necessary to have more information with regard to the amount of "normal" bone destruction that would be expected to occur in the absence of bone formation and the histologic appearances that this continued normal but unopposed bone destruction would produce

This element of uncertainty brings me to my second topic the recognition and assessment of the cellular processes responsible for bone formation and destruction, and I think that two important general questions can be posed in this connection. Is it possible morphologically to assess independently the sites and activities of these two processes? If this is so what information would such knowledge give us concerning the rate of turnover of bone tissue?

"Tissue Turnover" in Bone

The idea of a continuous "tissue turnover in bone goes back to the days of Duhamel and Hunter[31] when madder feeding experiments established the fact that bones grow by surface accretion This fact together with the knowledge that the marrow cavity of any given bone enlarges during its growth made Hunter realize that absorption of preexisting bone is a necessary accompaniment of growth Hunter you will recall ex

pressed the concept of the continuous "remodeling" of bone by the surprisingly modern comment that "living bone is constantly changing its matter" Tomes and de Morgan, in 1853[22] were aware of the implications of continuous remodeling processes in determining the arrangement of Haversian systems in adult cortical bone It was, however the researches of Goodsir[23] Koelliker[1] Virchow[25] and Gegenbaur[26, 27] that made it clear that bone formation resulted from the activity of a specialized cell, the osteoblast not long afterward one of the same workers[28] suggested that multinucleated giant cells or osteoclasts, were the agents by which bone destruction was brought about.

Quantitative Aspects. The processes of bone formation and bone destruction involve the surface of the mineralized skeletal tissue The surface to which I refer is not merely the external surface of a bone, but the more complex interface between the bone tissue and its ensheathing and included soft tissue components. It thus includes the surfaces of the trabeculae of cancellous bone, the periosteal and endosteal surface, and the linings of the many microscopic vascular channels which penetrate cortical bone It must not, however be confused with the overwhelmingly more extensive crystal surface of bone of which each of us, according to McLean and Urist,[29] has about 100 acres

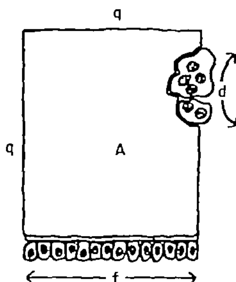


FIG. 1 13 Bone formation and bone destruction. *f* surface with bone formation in progress *d* surface with bone destruction in progress *q* inactive bone surface and *A* area of bone bounded by above surfaces.

Little attention appears to have been given to a consideration of the rates at which the processes of bone formation and bone destruction proceed or any possible relationship between these rates and the areas of surface involved Fig 1 13 summarizes these relationships for any given region of bone the areas *f* and *d* relate to the total amount of surface (diagrammatically lumped together for convenience) involved by bone formation and bone destruction respectively and *q* relates to the remaining inactive or "resting" part of the bone surface At any moment, if *F* and *D* are the rates of bone formation and bone destruction,

$$F = fg'$$

where g' is the rate of growth of new bone in thickness

$$D = dg''$$

where g is the comparable rate of erosion of bone in areas of destruction

For the normal adult the skeleton may be considered to be in balance so

$$F = D \qquad \text{and} \qquad fg' = dg''$$

i e., there is an inverse relationship between the linear rates of formation and destruction and the areas occupied by these processes

$$\text{Bone turnover} = \frac{100dg'}{A} \text{ \% per unit of time}$$

If we can measure the areas f d and A determination of one of the rates g or g' will allow us to calculate the other if the skeleton is in balance and we can also specify the rate of bone turnover for the area studied If the skeleton is not in balance independent assessment of the rates of bone formation and destruction (F and D) is possible if in addition we know either the second of the two linear rates or the change in the amount of bone tissue (A) that has occurred It is thus clearly of interest to know all we can of these rates and areas for normal bone tissue at different ages and in different situations and for various types of rarefying bone disease

At this point, a word is necessary with regard to the practical identification of the three components of the bone surface, for I have assumed hitherto that they can be distinguished from one another The view of most authorities (see for instance, Baker[30]) is that, in decalcified and stained bone sections, areas of bone formation can be identified by the demonstration of active osteoblasts on the surface concerned and by the presence immediately beneath these osteoblasts, of a thin layer of pale staining "osteoid" (see Fig 1 14) In compact cortical bone identification of such surfaces is aided by the fact that the Haversian systems concerned are incompletely formed in microradiographs they show a low density of calcification, and in experimental animals Engfeldt et al [31] have shown them to be the sites of maximum uptake of bone seeking radioactive isotopes such as Sr^{90} Ca^{45} and P^{32} In alcohol-fixed and undecalcified material Lacroix[32] and Vincent[33] have shown that the innermost layer of osteoid tissue gives a positive periodic acid–Schiff (PAS) reaction this is lost after formalin fixation and decalcification (see Meyer[34]) but in appropriately treated material it may aid the identification of areas of bone formation

Similarly areas of bone destruction can be identified by the presence of multinucleated osteoclast giant cells located on eroded bays in the bone surface (see Fig 1 15) The osteoclasts are often arranged in clusters The eroded bays are not infrequently more extensive than the accumulations of osteoclasts, and this has led to the suggestion that bone destruction can occur in the absence of these cells But it seems reasonable to assume with Baker[30] and Hancox,[35] that the osteoclast is a short lived cell and that after eroding bone it may quickly disappear leaving an irregular but quiescent area of bone surface There is some evidence according to Jowsey



Fig. 1 14 Normal bone. Outer part of cortex of femur in woman aged 57 years. Field includes a number of Haversian systems. One of these is in process of formation Its inner surface is lined by osteoblasts and shows an inconspicuous pale-staining border of osteoid tissue. (×175)

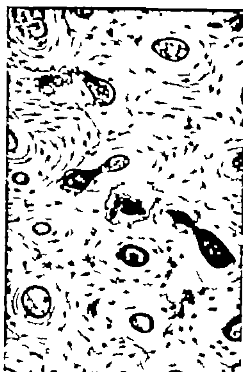


Fig. 1 15 Normal bone. Outer part of cortex of femur in woman aged 57 years. A small resorption cavity is present in the center of the field It has a sharply defined crenated outline and contains a cluster of osteoclasts. (×175)

et al [36, 37] that certain radioactive isotopes, such as Y^{91} show preferential uptake on the exposed bone surfaces where bone destruction is or has recently been, in action.

In adult bone however particularly in compact cortical bone it must be stressed that areas of bone formation and bone destruction are quite infrequent occupying only a small part of the bone surface the remainder of which is smooth and presumably quiescent (Fig 1 16)

In normal adult bone, the areas of osteoblastic bone formation are more extensive than those of osteoclastic bone resorption, from which we can infer that the linear rate of bone erosion by osteoclasts is more rapid than that of osteoblastic bone formation.

Returning to the expressions

$$F = fg' \qquad \text{and} \qquad D = dg'$$

we may assume then, that there are no insuperable problems with regard to the determination of the areas of the bone surface occupied by bone formation and bone destruction. Now we must consider the linear growth



FIG. 1 16. Normal bone. Inner part of cortex of femur in woman aged 57 years. Wide Haversian spaces with inactive or "resting" surfaces. (×80.)



FIG. 1 17 Exaggerated osteoclasis Inner part of cortex of femur in case of renal failure with slight bone changes Large resorption cavities are present, and a greatly increased part of the bone surface is occupied by osteoclastic bone destruction (×80.)

rates g' and g'' Until recently no information of any kind was available with regard to them. In any case knowledge of their magnitude depends on more information than is available in a tissue preparation, and any purely histologic assessment of the relative rates of bone formation and destruction (*F* and *D*) has consequently been based on the tacit assumption that these linear growth rates g' and g'' were constant Recently however the work of Vincent and Lacroix,[37] using radioactive isotopes as markers and of Vincent,[38] using lines of lead deposition for the same purpose allow the measurement of the rate of accretional growth g' at least for Haversian systems of cortical bone in experimental animals In the dog this quantity g' turns out to be about 10 μ/week Such a technique should make it possible to check, under experimental conditions, the importance of variations in the rates of linear bone formation and destruction (g' and g'')

While such variations in linear rates remain to be investigated, numerous observations testify to the importance of changes in area in connection with both osteoblastic and osteoclastic activity In Fig. 1 17 for example from

a case of renal failure with slight bone changes, we see an area of cortical bone where some large resorption cavities have developed and where a considerable part of the bone surface is occupied by osteoclastic resorption This is the exaggerated osteoclasis" of descriptive histology and it is just this extension of osteoclasts to an abnormally large part of the bone surface that has not been encountered in Cushing's syndrome. Whether it could be distinguished from normal would of course, depend on its magnitude and on the amount of normal material available for comparison here again, I want to stress the need for the quantitation study of osteoblastic and osteoclastic activity at various skeletal sites in normal bones and in various types of generalized rarefying bone disease *



FIG 1 18. Exaggerated osteoblastic and osteoclastic activity Outer part of cortex of femur in case of primary hyperparathyroidism with severe bone changes. Large resorption cavities are present, and the bone surface is almost entirely covered by areas of osteoblastic bone formation and osteoclastic bone destruction. (×80)

In Fig 1 18 an area of rarefied cortical bone from a case of primary hyperparathyroidism with severe bone changes, not only is there an extension of osteoclasis, but a large part of the bone surface is covered by osteoblasts and newly formed bone On the basis of area both bone formation and bone destruction appear to be occurring at a greatly increased rate, and we are dealing with an area of rarefied bone where the rate of tissue turnover is high

Other Evidence of "Bone Turnover" The concept of "bone turnover" that I have outlined is not based solely on morphologic considerations Today a number of studies on topics such as the skeletal distribution of radioactive isotopes by Leblond et al.,[39] Comar et al [40] Tomlin et al [41] Jowsey et al [42] Owen et al [41] Vincent,[37] and Arnold et al.[41] and the excretion of calcium and lead in cases of lead poisoning by Bauer et al.[43]

* As a working hypothesis, the ratios $f/(f+d+q)$ and $d/(d+f+q)$ might be taken as a measure of the activity of bone formation and bone destruction in the absence of any observations where g' or g can be directly measured. (In comparing different sites and individuals, these ratios would appear preferable to f/A and d/A which fail to take into account differences in the structural arrangement of the tissues.)

or of radioactive materials by Norris et al[46] all confirm the concept and give some idea of its magnitude In this connection I am particularly aware of the stimulating approach of Bauer[47, 48] and his colleagues which attempts to use the rates of skeletal uptake of radioactive isotopes and the relationship between the specific activities of the skeleton and the circulating blood to determine rates of bone formation and bone destruction I think however that the uncertainty of the anatomic meaning of the various isotope "compartments" of the skeleton and the difficulty of distinguishing between "exchange" reactions and actual tissue formation and destruction make it desirable to have alternative and more direct, ways of evaluating bone turnover The approach I have outlined, moreover is applicable to particular sites in the skeleton while local differences in behavior are lost in studies of over all isotope uptake or excretion

I have not been able on this occasion to follow these ideas on the quantitative aspects of osteoblastic and osteoclastic activity to their logical conclusions but I hope that I have been able to show you some of the possibilities—as well as some of the limitations—of a morphologic approach to the problem of bone turnover and their bearing on our knowledge of the local cellular mechanisms concerned in the development of osteoporosis In particular I hope I have been able to indicate that in 1958 just 100 years after Virchow's founding of cellular pathology a consideration of cells as functional units still has interest and application in the particular field of research with which we are all concerned

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Observations Bearing on the Problem of Osteoporosis*

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Osteoporosis defined as loss of bone mass increased bone porosity and decrease in thickness of cortex in humans, with normal levels of serum calcium phosphorus and alkaline phosphatase with normal mineral and vitamin intake, and with negative calcium phosphorus, and nitrogen balance is an unsolved problem. There are four main theories of the etiology of osteoporosis dietary deficiency of calcium achlorhydria lowering calcium absorption, dietary deficiency of protein and gonadal hormone deficiency The condition is generally regarded as idiopathic, if an endocrine gland disorder cannot be found and if relief of pain (or positive calcium balance) cannot be produced by treatment with sex hormones At the present time there is no specific laboratory test that can distinguish patients with osteoporosis from patients of comparable age or illness without osteoporosis hence all cases, either before or after the menopause, could be classified as idiopathic osteoporosis This communication will present a summary of clinical and laboratory studies of cases with fractures that required orthopedic measures as well as cases that were undiagnosed and asymptomatic in an attempt to outline the broad scope of the problem of osteoporosis

Case Material, Methods, and Results. The records and x ray films in the archives of the following institutions were examined to find 837 patients (Table 2 1) described in this communication United States Veterans Administration Medical Center Domiciliary Wadsworth Hospital Brent

* These investigations were aided in part by grants from the Josiah Macy Jr Foundation, the Ayerst Laboratories, Inc., the Lee S. Mylinger Foundation, and the Easter Seal Foundation for Crippled Children and Adults.

Table 2 1

SELECTION OF CASES

No case	Table no	Classification	Per cent osteoporosis
100	2 2	Ambulatory (chronically ill) domiciliary men)	18
100		Epileptic men	30
100	2–3	Healthy" aged women	20
100	2–4	Women with fracture of the hip	76
25	2–5	Women with ununited fracture of the hip	81
100	2–6	Men without osteoporosis	0
100	2–6	Men with osteoporosis	100
100	2–7	Women without osteoporosis	0
100	2–7	Women with osteoporosis	100
12	2–9	Addison s disease	0

wood Sanitarium and the Medical Center University of California, Los Angeles and the Eastern Star Home West Los Angeles, California

Diagnosis, Radiographic Considerations, and Selection of Cases

The diagnosis of the patients described in this communication was based chiefly on roentgenograms of the spine in the lateral view and the finding of the following five pathologic changes (1) collapse of one or more vertebral bodies of the dorsal spine (2) biconcave vertebra in the dorsal spine (3) reduction of the thickness of the cortex of the vertebra to at least one-half of that seen in patients without osteoporosis (4) resorption of the horizontal trabeculae and accentuation of the vertical trabeculae of the cancellous bone of the bodies of the vertebra and the neck of the femur and (5) absence of appreciable spondylosis or annular osteophytes, or bone spurs Patients without collapsed vertebra (and some of the other radiographic changes enumerated above) were arbitrarily excluded from this study It was assumed accordingly that these gross criteria disclosed the minimum number of cases of osteoporosis and did not include either cases in the early stages of the disorder or rare cases of hyperparathyroidism or osteomalacia (which are relatively few and recognizable by laboratory and biopsy examinations)

The distribution of the collapsed vertebra and fractures in the patients with the least severe compared with patients with the most severe degree of osteoporosis indicated that the disorder generally begins in the thoracic spine and involves the ribs lumbar spine pelvis and hips later In the thoracic region, because of the dorsal curve of the spine the stress was on the vertebral bodies and response to resorption of the cortical bone plates

was collapse of the bone structure In the lumbar region, because of the anterior curve of the spine, the stress was on the posterior border of

Table 2 2

CONDITION OF THE SPINE IN 100 CONSECUTIVE MEN (AVERAGE AGE 66)

Radiologic observations	*Per cent*
Generally negative, good bone density	9
Spondylosis, good or increased bone density	70
Osteoporosis, diagnosis based upon collapsed vertebra	18
Compression fractures of the dorsal spine	27
Biconcave lumbar vertebra	14
Compression fractures of the lumbar spine	15
Compression fractures of both dorsal and lumbar spine	9
Spondylosis (minimal) and osteoporosis	10
Severe kyphosis with spondylosis	3
Severe kyphosis, osteoporotic (from fractures)	11
Severe kyphosis Scheuermann s type	0

the vertebral bodies and the facets, and strain was brought to bear on the intervertebral discs. This spared the vertebral bodies from collapse but resulted in remodeling of the vertebral end-plates and expansion of the intervertebral discs, causing biconcave vertebral bodies (so-called "codfish-shaped" vertebra of Albright)

The Incidence of Osteoporosis

Incidence in Men. The incidence of osteoporosis in "healthy" men is not known. In a series of ambulatory men with various chronic illnesses such as arteriosclerosis, emphysema, hypertension, and syphilis, and in residence at the Veterans Domicillary the incidence of fractures and advanced osteoporosis was 18 per cent. There was an additional 6 per cent that could be classified as osteoporosis but were excluded because the condition was not at an advanced stage In nonhospitalized men the condition was relatively asymptomatic and limited to the spine. Few of these men patients had fractures of the ribs or hips or suddenly collapsing vertebra, or acute episodes requiring hospitalization The x ray changes gave the impression that senile osteoporosis in aged men is more slowly progressive and reaches the most advanced stages of the condition less frequently than in women The greater longevity of women may or may not, be a factor in this connection, but in general these findings in both men and women are comparable to those reported by Gershon-Cohen et al [1]

Incidence of Osteoporosis in 100 Men with Chronic Epilepsy The average age of the patients in this group was 60 1 and all had a record of innumerable grand mal seizures since early adult life The incidence of compression fractures of the dorsal spine was 30 per cent. The incidence of thinning of the cortex and biconcave vertebra in addition to the fractures

was 16 per cent Further study of these patients is necessary to determine whether there was some predisposing endocrine or nutritional condition such as osteoporosis that was responsible for the fractures The fact that 70 per cent had an equal number of seizures and equal exposure to trauma and did not show fractures or osteoporosis requires investigation, the frequent occurrence of fractures in epileptics is well known but the subject seems not to have been considered in relation to osteoporosis.

Incidence in Women. Table 2 3 summarizes the results of a radiographic survey of the spine of 100 aged women. The minimum number of cases of osteoporosis in this group was 26 per cent. More than 60 per cent, however showed normal and even increased bone density with varying degrees of spondylosis but no collapsed vertebra. These patients suggested that osteoporosis is not a natural concomitant of aging Fourteen per cent showed good bone density with neither spondylosis nor osteoporosis. This group may include an unknown number of individuals that could conceivably develop osteoporosis and collapsed vertebra at a later age. Approximately 12 per cent showed osteoporosis and spondylosis in the same patient and it was very rare for severe osteoporosis to appear in spines with large spurs or annular osteophytes Serial roentgenograms on file over a period of 10 years in some cases gave the impression that osteoporosis and hypertrophic spondylosis are separate and distinct disorders requiring a long period of time to develop and caused by diametrically opposite conditions The incidence of fracture of the hip was 15 per cent in this group

Table 2-3

CONDITION OF THE SPINE IN 100 CONSECUTIVE WOMEN (AVERAGE AGE 85)

Radiologic observations	*Per cent*
Generally negative good bone density	14
Spondylosis, good or increased bone density	60
Osteoporosis (diagnosis based upon collapsed vertebra)	26
Compression fractures of dorsal spine	26
Biconcave lumbar vertebra	24
Compression fractures of lumbar vertebra	20
Compression fractures in both dorsal and lumbar spine	18
Fractures of the hip	15
Spondylosis and osteoporosis	12
Severe kyphosis with spondylosis	4
Severe kyphosis, osteoporotic (from fractures)	14
Severe kyphosis, Scheuermann s type	1

Incidence in Women with Fractures of the Hip. Table 2-4 summarizes the results of a radiographic survey of the spine in 100 aged women admitted to the orthopedic service for a fracture of the hip Only 28 per cent recalled having had low back pain, but 76 per cent had advanced osteoporosis of the spine Of the fractures of the hip 50 per cent were

Table 2-4

100 CONSECUTIVE FRACTURES OF THE HIP IN WOMEN AGES 40 TO 90
(AVERAGE AGE 73)

Summary of records	*Per cent*
History of a trivial degree of injury	94
Type of fracture	
Intertrochanteric	50
Midcervical intracapsular	18
Subcapital, intracapsular	32
Nonunion, over all incidence	26
Incidence of nonunion in intracapsular fractures	44
Past history of low back pain	28
Preexisting osteoporosis with collapse of dorsal vertebra or biconcave lumbar vertebra	76
Both forms of change in spine	60
Reambulation within 1 year unaided by walker	50
Deceased within 1 year	18

intracapsular and 50 per cent were extracapsular The mechanism of injury in 94 per cent was such trivial incidents as stumbling, sudden twisting to rise out of a chair or hastening to answer the telephone In most cases *it was difficult to decide whether the fall came before or after the patient* felt pain and incurred the fracture For this reason, many observers believe that the majority of fractures of the hip in the aged are spontaneous or pathologic fractures.

The condition responsible for the fracture judging from the roentgenograms of the opposite or sound hip in this series, was osteoporosis. The superior and lateral cortex of the upper end of the femur was less than half of that seen in patients of similar age without osteoporosis. The horizontal trabeculae of the cancellous bone in the interior of the neck of the femur were resorbed some of the vertical and diagonal trabeculae were also resorbed this appeared to increase the thickness and accentuate the structure of the remaining vertical trabeculae enough to permit counting them The number of trabeculae in the trajectorial pattern of the spongiosa inside the neck of the femur was reduced from 35 to 45 to as few as *10 to 15 By such trabecular counts it was possible to predict accurately* when a patient would also have collapsed vertebra in lateral roentgenograms of the spine

Of the cases summarized in Table 2-4 24 per cent did not have osteoporosis of the spine Many of these patients had a severe slip and fall downstairs or an injury from an automobile accident. Others had disuse atrophy of one or both extremities from preexisting paralytic conditions. There was no reason to assume that osteoporosis of the spine could occur from disuse simply by immobilization of the patient in bed.

Incidence in 25 Consecutive Ununited Fractures of the Hip. Table 2 5 summarizes the results of a radiographic survey of the spine in 25 aged

Table 2

CONSECUTIVE SUBCAPITAL FRACTURES WITH POOR END RESULTS OF NAILING OPERATIONS

Summary of findings	*No*	*Per cent*
History of trivial injury	21	84
Nail protruding into acetabulum	11	44
Extrusion of nail from the neck	9	36
Absorption of the neck	[illegible]	68
External rotation contracture	[illegible]	100
Avascular necrosis of the head	1	4
Fracture reduced and nail well placed	12	48
Fracture not reduced or imperfect nailing	13	52
Compression fractures osteoporosis of dorsal spine	21	84
Codfish vertebra lumbar spine	[illegible]	60
Compression fractures lumbar spine	10	40
Combination of above three changes	9	36

women with unsuccessful results from operations to provide internal fixation for a fracture of the hip The incidence of osteoporosis was 84 per cent. While the number of cases is too small to be significant some important impressions were obtained from this group of patients Osteoporosis was not itself the cause of nonunion it was the chief cause of failure to secure internal fixation of the fracture Only one fracture failed to unite because of avascular necrosis of the femoral head But 52 per cent failed to unite because the fracture was not reduced in many of these the brittle bone of the neck broke into small chips and splinters that constituted missing bone substance at the fracture line In several of these cases the operative technique was faulty for reasons that were not obvious In 48 per cent, the fracture appeared to be reduced and the nail well placed but there was insufficient cancellous bone substance in the neck or head to support the nail Presumably for this reason the nail either migrated forward into the acetabulum in 44 per cent or backward out of the neck in 36 per cent

It was concluded that a radiographic examination of the spine should be made in all patients with fractures of the hip in order to anticipate the factor of osteoporosis as an obstacle to achieving good internal fixation. The trend in recent years to introduce changes in the design of the nails used for internal fixation appears to be the wrong approach to the unsolved problem of fracture of the neck of the femur The problem appears not to be in the nail but in the brittle osteoporotic bone tissue Furthermore the choice of treatment must be influenced not only by the mechanical problem but also by the pathologic process in progress in the individual patient

Hormonal and Neuroendocrine Disorders in Men with and without Osteoporosis

In order to analyze endocrine factors a list was made of the various acute and chronic disorders found in 100 osteoporotic and 100 nonosteoporotic individuals. The list of diagnoses associated with osteoporosis in each case was obtained from the summary of the hospital charts. We found 100 consecutive cases in approximately 120 000 men patients admitted to the hospital during the period from 1948 to 1958 Arteriosclerosis, hypertension chronic pulmonary disease degenerative joint disease, and syphilis were equally common in osteoporotics and nonosteoporotics The outstanding difference of the two groups was the 18 per cent incidence of duodenal ulcer (possibly a neuroendocrine disorder) in patients with osteoporosis The similarity in the two groups, however was more striking and statistically more significant the incidence of both intestinal ulcers plus endocrine disorders was 29 per cent in the osteoporotics but as high as 15 per cent in the nonosteoporotics (Table 2-6)

Table 2-6

HORMONAL DISORDERS ESTABLISHED IN 100 MEN WITH AND 100 MEN WITHOUT OSTEOPOROSIS (AVERAGE AGE 61)

Hormonal disorder	*Osteoporosis*	
	Per cent without	*Per cent with*
None	85	71
Duodenal ulcer	0	18
Diabetes	6	4
Renal calculi	0	3
Hypogonadism	0	2
Hyperthyroidism	0	2

Hormonal and Neuroendocrine Disorders in Women with and without Osteoporosis

Of 100 consecutive women patients with osteoporosis, endocrine disorders were found in 22 per cent, consisting of ovariectomy in only 10 diabetes in 4 and other forms in 8 In 100 women either ambulatory or bedridden without osteoporosis of the spine, endocrine disorders were present in 6 per cent. Three had had ovariectomy and yet did not have osteoporosis. The diagnoses appearing in the records most frequently in both osteoporotic and nonosteoporotic groups were arteriosclerosis hyper tension, degenerative joint disease and malignancy It was interesting that symptomatic duodenal ulcer a relatively uncommon condition in women

in the urine than the aged both before and after prednisone treatment, but they probably turned over more calcium and may have had much less skeletal loss than aged individuals. Radioisotope studies with Ca^{47} are necessary to investigate these points further.

Another effect of adrenal steroids that may have a bearing on the development of osteoporosis is the suppression of gonadal function. The patients listed in Table 2-8 invariably showed such evidence of suppression of gonadal function as loss of libido and testicular atrophy. Observations by the author on birds (animals which have an extremely sensitive adrenogonadal endocrine system) revealed that ACTH and cortisone treatment reduced the size and weight of the ovaries as much as 75 per cent and halted egg production. The urinary excretion of 17 ketosteroids (17KS) and estrogen was not measured but this should be done both experimentally and clinically to determine whether suppression of gonadal hormone production and adrenogonadal imbalance must inevitably follow cortisone treatment.

Addison's Disease

The roentgenograms of the spine were examined in 12 patients with Addison's disease (Table 2-9). Two of the patients with bone metastases

Table 2-9

IS OSTEOPOROSIS POSSIBLE IN ADDISON'S DISEASE?

Cause	*No. patients*	*Age*
Idiopathic	4	30-65
Cushing's syndrome	2	15-35
Carcinoma of breast	2	52-60
Carcinoma of prostate	4	60-74

had collapsed vertebra, but there was no osteoporosis. This raised the question of whether it is possible for osteoporosis to occur under conditions of low 11-oxy 17-hydroxycorticosterone (17OHCS) in the plasma. The so-called "permissive" action of the adrenal cortex as interpreted by Ingle[3] suggests that 17OHCS may be as necessary to produce osteoporosis as it is to produce diabetes. A larger sample of Addisonian patients and patients with panhypopituitarism should be studied to answer this question.

Dietary Intake of Calcium

A survey was made of the dietary habits of 100 aged women with a 26 per cent incidence of osteoporosis, with special reference to the content of calcium and dairy foods. It is well known that milk and cheese are the

chief source of calcium in the diet in the United States Apparently there is no food or drug that yields calcium to man as well as milk It is also impossible to meet the daily average requirement of 800 mg of calcium without milk or cheese or a similar dairy product in the diet

Table 2 10 summarizes the observation that 21 per cent of "healthy

Table 2 10

DIETARY INTAKE OF CALCIUM IN 100 AGED WOMEN (AVERAGE AGE 85)

Daily average intake mg	*Per cent*
Less than 200	21
200–800	76
More than 800	3

aged women existed on a diet seriously deficient in calcium (200 mg or less per day) because of a personal aversion to dairy foods, or the belief that milk caused distention or constipation, or the conviction that they were allergic to milk and milk products. Only 2 of these 21 women had osteoporosis Twenty-six women who had osteoporosis (Table 2 11) se-

Table 2 11

DIETARY INTAKE OF CALCIUM IN 20 WOMEN WITH SEVERE OSTEOPOROSIS

Daily average intake mg	*Per cent*
Less than 200	8
200–800	85
More than 800	7

lected a diet that varied widely in calcium content, only 8 per cent of this group had a seriously deficient calcium intake and 7 per cent were actually on a very high calcium intake because of a fondness for dairy products of all kinds Eighty four aged women who had no osteoporosis (Table 2 12) also showed a widely variable daily intake of calcium, but

Table 2 12

DIETARY INTAKE OF CALCIUM IN 84 AGED WOMEN WITHOUT OSTEOPOROSIS

Daily average intake mg	*Per cent*
Less than 200	18
200–800	79
More than 800	3

it was significant that as high as 18 per cent selected a diet that is generally regarded as grossly deficient in calcium These observations suggest that it is doubtful if in the United States, low calcium intake is primarily

responsible for osteoporosis or that high calcium intake prevents osteoporosis. Rarely can fault be found with calcium absorption because patients with senile osteoporosis generally have been found to show positive calcium balances when exposed to increasing levels of calcium intake The body seems to be able to adjust to some extent to high or low calcium intake and sustain equilibrium between blood and bone even in aged individuals.

Laboratory Data

Serum Calcium Phosphorus, and Alkaline Phosphatase. By definition, osteoporosis is generally regarded as a condition in which the levels of the components of the blood are within normal limits Patients whose records showed other findings were not included in this investigation.

Serum Protein. The levels of the plasma proteins were determined chemically and electrophoretically in 10 patients with severe osteoporosis and 20 patients of comparable age without osteoporosis An ultracentrifugal analysis, including the low and high-density lipoproteins was made of the serum of 4 patients in each group The total protein levels of the serum were the same in osteoporotic and nonosteoporotic individuals, varying from 6 1 to 7 0 Gm per 100 cc the patients in the eighth and ninth decades, however showed lower levels than younger individuals, chiefly because of lower levels of the albumin fraction. Here the protein levels *varied from 2.9 to 3.5 Gm per 100 cc. In any case, low serum albumin* levels could not be regarded as a specific characteristic of osteoporosis

Serum Lipoproteins. The low and high-density lipoproteins were within the limits of normal for the age of the individual In general, S_f classifications 0–12 were 319 to 619 the S_f 12–400 were 59 to 294 mg per 100 cc, and both were higher in individuals of advanced age than in individuals of middle age regardless of the incidence of osteoporosis. This was also *true of the atherogenic index of Delalla, Elliot, and Gofman*[3] Estrogen treatment increased the level of the alpha lipoproteins and decreased the beta lipoproteins in osteoporotic patients to the same extent as in nonosteoporotic patients

Urinary Calcium Excretion. The amount of calcium excreted in the urine over a period of 24 hours was determined in 104 patients from all groups (Tables 2 1 to 2 8) The level in patients with or without osteoporosis on *unselected diets was highly variable with a range of 50 to 400 mg* 30 per cent of the patients with osteoporosis showed lower levels 40 to 100 mg than patients of similar age without osteoporosis. One patient with such severe osteoporosis that she was bedridden excreted less than 40 mg Such a level was common in aged individuals in whom the osteoporosis was so extreme that their skeleton was nearly depleted of the labile calcium stores.

Metabolic Balance Studies. Four patients had been selected for metabolic balance studies and showed either normal or low calcium, phosphorus, or

nitrogen retention Positive balances were obtained in all four during brief test periods on sex hormone therapy

Plasma 17OHCS. The level of the nonconjugated (free and protein bound) 11-oxy 17 hydroxycorticosterone of the plasma was measured by the method of Nelson and Samuels[4] in 10 women with severe osteoporosis and in 14 women of comparable age without osteoporosis In both groups, the levels ranged from 10 to 16 μGm per 100 cc, and all cases showed levels within limits regarded as normal

Spinal Fluid 17OHCS. In one patient with severe osteoporosis (Case 1) the spinal fluid was analyzed for 17OHCS and found to contain 2 μGm per 100 cc this is within the limits of the range found in normal subjects

Urinary Excretion of 17OHCS. The quantity of 17OHCS excreted in the urine every 24 hours was measured in 12 women and 4 men with osteoporosis and 10 women and 2 men of comparable age without osteoporosis by the following two methods the Norymberski technic[5] and the method of Glenn and Nelson.[6] The amount of 17OHCS and ketogenic steroids measured by the Norymberski technic varied from 8 to 25 mg in men and 5 to 18 mg in women in both the osteoporotic and nonosteoporotic cases the amount of the 17OHCS measured by the Glenn and Nelson method varied from 5 to 8 mg In 5 cases of women with osteoporosis and 5 cases of women without osteoporosis, the urinary 17OHCS was determined as both free and conjugated The free 17OHCS was the same in both groups and varied from 0 1 to 0 4 mg The conjugated 17OHCS after hydrolysis with glucuronidase varied from 0 9 to 7 8 mg The patients with severe osteoporosis excreted 5 8 to 7 8 mg of the conjugated form larger amounts than the controls, but these were st ll less than may be expected of some normal women and most patients with Cushing s syndrome

Diurnal Variation in the Excretion of 17OHCS in Urine. Specimens of urine were collected in two samples one during the day and one during the night, in 2 osteoporotic and 2 normal patients over a period of 24 hours in order to measure the diurnal cycle of excretion of 17OHCS Laidlaw et al.[7] observed that some patients with Cushing s syndrome had normal levels of 17OHCS excretion but differed from normal control individuals in that the adrenal cortical hormone metabolites were excreted largely during the day in normal individuals but in equally large amounts during the day and night in patients with Cushing s syndrome One patient with osteoporosis (similar to the patients with normal spines) excreted 2 2 to 4 6 mg during the day and only 0 9 to 2 8 during the night The other patient had had a reversal of the diurnal cycle and excreted more during the day than during the night.

Urinary Excretion of 17KS. The quantity of the 17 ketosteroids excreted in the urine in 24 hours was measured by the method of Sobel et al [8] in 24 women and 2 men with osteoporosis and 12 women and 12 men without osteoporosis. The range of the age of the patients was from 61 to 85 years

The excretion of 17KS was generally lower in the aged than in premenopausal adults, but it was the same in the osteoporotic and the nonosteoporotic cases and varied from 2.7 to 4 1 mg in 24 hours. This amount was found to rise to 5 6 to 6 0 mg following gonadal hormone therapy but not so high as the 6 0 to 15 mg as found in premenopausal individuals

Urinary Excretion of Estrogen. The quantity of estriol, estrone, and estradiol excreted in the urine in 24 hours was measured by the method of Brown[9] in 15 women with osteoporosis and 5 women without osteoporosis. The range of the age of the patients was from 48 to 87 years. The estrogen excretion of the older age women was significantly lower than that of the younger age premenopausal women, but there were no appreciable differences in the osteoporotic and nonosteoporotic groups, and the levels varied from 5 to 9 μGm for estriol, 4 to 17 μGm for estrone 2 to 8 μGm for estradiol, and 9 to 40 μGm for total estrogen in 24 hours. Administration of estrogenic substances either oral or parenteral, promptly raised the level of estrogen excretion from the high value of 40 μGm before treatment to 200 to 300 μGm after treatment.

Special Tests

ACTH Capacity Test. Three patients with severe osteoporosis received an infusion of 40 units of ACTH over a period of 2 hours, and samples of their plasma were analyzed for 17OHCS at 1-hour intervals over a period of 6 hours to determine the capacity of the adrenal cortex for production of glucocorticoid hormones The peak level of production was slightly if at all different from that of normal individuals of comparable age The rate of fall in the plasma 17OHCS however in one case was somewhat slower and suggested a highly active adrenal cortex (Fig 2 1)

Cortisone Metabolism Test. Three patients with severe osteoporosis were given an infusion of 80 mg of cortisol over a period of 2 hours, and samples of their plasma were analyzed for 17OHCS over a period of 6 hours to determine the rate of removal of the hormone from the blood. This has been regarded as a test of the ability of the liver to transform active 17OHCS (free) into inactive conjugates (chiefly glucuronides) Rather than being impaired, in one patient with osteoporosis free cortisol was removed from the plasma more rapidly than in nonosteoporotic patients of comparable age (Fig 2 1)

Prednisone or Cortisol Calcium Excretion Test. The excretion of calcium in the urine was measured in 10 patients with severe osteoporosis and 10 patients without osteoporosis Normally one-fourth of the total daily calcium excretion occurs from the urinary route and three fourths from the fecal route Inasmuch as the calcium excreted by the intestine is largely unabsorbed from the diet the urinary calcium gives an adequate indica-

tion of the amount mobilized daily from the skeleton under the influence of hormonal factors.

Figure 2 2 (patterned after the chart by Irwin et al [10]) illustrates the observation that aged individuals with severe osteoporosis on unselected

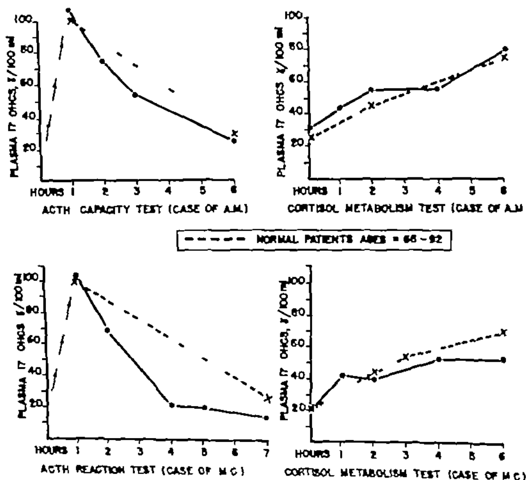


FIG 2 1 Four charts showing the results of ACTH capacity test and cortisol metabolism test in typical examples of symptomatic (Case 1 A.M) and asymptomatic osteoporosis (Case 2, M C.) of the spine with fractures of the neck of the femur In both instances an intravenous infusion of 40 units of ACTH produced a prompt rise and a fall in the level of the plasma 17OHCS that was slightly more rapid than in nonosteoporotic individuals of comparable age In both instances, 80 mg of cortisol infused intravenously over a period of 6 hours was removed from the plasma at approximately the same rate as normal.

diets excrete only 50 to 75 mg of calcium, only one half to one fourth as much as individuals without osteoporosis On a low calcium control diet, this amount was generally unchanged and not reduced further With cognizance of this fact, the patients were observed while receiving unselected diets containing 200 to 800 mg of calcium per day Prednisone 20 mg per

day was administered orally over a period of 5 days. The patients with osteoporosis generally showed a 75 to 100 per cent increase in excretion of urinary calcium The patients without osteoporosis showed a highly vari

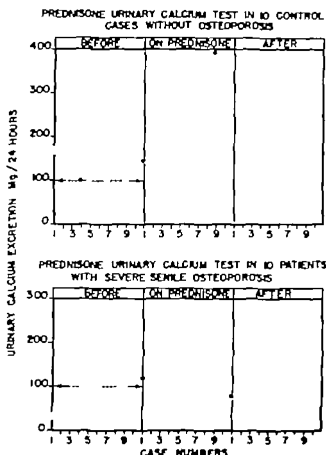


FIG. 2 2. Six charts showing the changes in the level of the 24-hour urinary calcium in 10 patients with severe osteoporosis of the spine and 10 patients without osteoporosis, before, during, and after recovery from 20 mg of prednisone per day for 5 days. The patients with severe osteoporosis excreted less than 100 mg of calcium per day often as little as 40 mg, and showed a slight diuresis with a threefold increase in calcium excretion in response to prednisone The patients without osteoporosis, aged as well as young premenopausal individuals (cases 9 and 10) always excreted more than 100 mg of calcium daily and showed diuresis with no increase or a relatively slight increase in calcium excretion after similar prednisone treatment. Both osteoporotic and nonosteoporotic subjects showed an irregular but definite return to the previous daily level of calcium excretion 1 week after the steroid was discontinued.

able response Some individuals showed no change others showed a 30 per cent to 100 per cent increase others showed a decrease in the amount of calcium excreted in the urine in 24 hours. None of the patients with severe osteoporosis showed such effects as either retention or no loss of

calcium Did prednisone accentuate an endocrine imbalance that was already present in patients with osteoporosis?

The observation that calcium most likely from the skeleton is excreted in the urine in large amounts in response to prednisone would seem to indicate that adrenal steroids did accentuate the disorder and is strong evidence in favor of the endocrine theory of the origin of osteoporosis Because the physiologically active metabolite of prednisone is apparently 17OHCS or cortisol the urinary calcium response was measured in one osteoporotic patient following a direct infusion of cortisol Cortisol 80 mg infused intravenously over a period of 4 hours raised the total urinary calcium excretion from 70 to 160 mg per 24 hours Cortisol 160 mg, raised this level further to 190 mg per 24-hour period The level of urinary calcium excretion in this individual gradually returned to its previous low level over a period of 1 week

Since the quantity of calcium excreted in the urine was proportional to the amount of cortisol it may be assumed that the effect was due to the action of adrenal corticoids upon the skeleton The possibility that this effect may be upon kidney function can be excluded by the experiment of Grollman,[11] who found hypercalcemia in cortisone treated nephrectomized dogs.

It is interesting to speculate on the possibility that patients who do not have osteoporosis are able to protect their bones against the bone resorbing action of the 17OHCS output of the adrenal It is well known that estrogen lowers the excretion of calcium in the urine in cortisone-treated patients or patients with Cushing s disease as well as in patients with senile osteoporosis. The mechanism of this bone-sparing effect is not known Szego observed that estrogen competes with 17OHCS for the limited amount of the plasma protein that is capable of transporting steroid hormones to their target tissues. Mills[12] found that estrogen stimulates the production of a special "interband" globulin that binds 17OHCS in an inactive form in the plasma until it is conjugated and excreted in the urine

Sample Cases and Cortical Bone Biopsies

Twenty of the women and two of the men patients described above were hospitalized on orthopedic wards for spontaneous and traumatic fractures of the hip Two patients were referred from medical to orthopedic sections of the hospital for biopsy examinations Three case histories are summarized here because they are typical and illustrative of many of the others described in this report The biopsy specimens were obtained from the cortex of the lateral aspect of the upper end of the femur 3 to 5 cm distal to the externus tubercle The operations were performed in conjunction with open reduction and internal fixation of the fracture except when

the procedure was advised for diagnostic purposes only Biopsy studies in the past have emphasized the examination of cancellous bone The changes in the cortical bone structure of the following three patients were more typical of osteoporosis than any other condition of the skeleton and are pathognomonic of this disorder

CASE 1 A.M. **Senile Osteoporosis with Presenting Symptoms Beginning in Spine** A 78 year-old retired Army nurse was hospitalized periodically from 1945 to 1958 for low back pain, fractures of the spine, ribs, and hip renal calculi and pyelonephritis. The *past medical history* was extraordinarily unremarkable. The patient had no pelvic operation or evidence of any of the endocrine diseases regarded as causes of osteoporosis. Physical examinations revealed a pale, somewhat senile woman who had lost 8 in. in height and 30 lb in weight. There were three deep transverse abdominal folds. The spine showed marked dorsal kyphosis and flattening of the normal anterior lumbar curve. The patient was ambulatory until March 3 1958 when she incurred a spontaneous intertrochanteric fracture of the left hip The laboratory findings of special interest were serum calcium 9 6 mg per 100 cc· serum phosphorus, 3 3 mg per 100 cc· alkaline phosphatase, 3.2 units total protein, 7 13 Gm per 100 cc albumin, 3.3 Gm per 100 cc· globulin 3 83 Gm per 100 cc; A/G ratio 0.87 Bence Jones protein was absent in the urine the 24-hour urinary calcium excretion was only 32 mg The results of special tests for various hormone factors were as listed in the section on laboratory data of this report. The x ray examination showed typical advanced osteoporosis with collapse *of all the vertebral bodies of the dorsal spine*, extreme *"ballooning"* of the lumbar intervertebral discs, and reduction of the thickness of the cortex of all the bones of the spine, shoulder and pelvic girdles (Fig 2 3) Biopsy and an adrenalectomy were performed in 1958 and found to show atrophy fasciculata and reduction of the weight of the glands to one-half of normal The right adrenal weighed only 4 Gm, the left 6 Gm and showed normal thickness of the zona fasciculata but marked thinning of the zona glomerulosa and the zona reticularis.

CASE 2. M.C. **Senile Osteoporosis with Failure of Internal Fixation of a Fracture of the Hip as the Presenting Sign** A 75-year-old housewife felt a sharp pain in the hip and fell while stepping off a curbstone. She said she had previously been in good health, except for slight swelling of the ankles necessitating the use of digitoxin for a "heart condition." She denied ever having had a backache. She had no recognizable endocrine disorders and had not had surgical removal of her ovaries. The physical examination and the laboratory findings were essentially the same as those found in Case 1 The patient had a slight hypoproteinemia due entirely to a low level of the albumin fraction, but did not have renal calculi or genitourinary tract infection. The x-ray examination showed collapse of *the vertebral bodies of the 8th, 9th, and 11th dorsal vertebrae and* ballooning of the intervertebral discs, thinning of the cortex of all the lumbar vertebrae, and a midcervical fracture of the right femur A specimen of bone was removed from the lateral cortex of the



D *E*

F

Fig 2 3 *D* Roentgenogram of the pelvis and both hips of the same case as shown in *A* to *C* Note the accentuation of the vertical trabeculae (indicated by the arrow) of the spongiosa of the internal architecture of the neck of the femur The horizontal trabeculae, including those of remnants of the old epiphyseal line, were resorbed. *E* Roentgenogram of the left hip of the same patient as shown in *A* to *D* following a fracture of the hip that was incurred when the patient merely rolled over in bed in the hospital The biopsy specimen in *F* was removed from the area indicated by the rectangle, in conjunction with an open operation and excision of the head and neck of the femur *F* Photomicrograph showing a histological section of the cortex of the femur from the area shown in the rectangle in *E* showing fatty fibrous marrow and no osteoid borders.

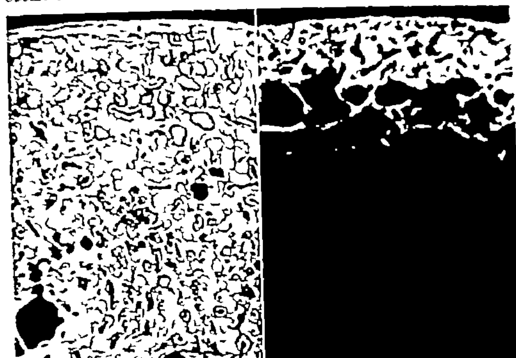




FIG 2-4 *A* Histologic preparation of a cross section of the lateral cortex of the shaft of the femur in a patient with Cushing's syndrome and osteoporosis due to prolonged treatment with prednisone. Note the absence of osteoid tissue and large resorption cavities on endosteal surface of the cortex. Hematoxylin and eosin ×15 *B* Microradiograph of a serial section as shown in *A* resorption of the endosteal surfaces of the cortical bone and variable areas of new and old lamellar bone with high- and low-density calcification. *C* Microautoradiograph of the sections shown in *A* and *B* following an intravenous infusion of P^{32} as sodium phosphate, 1 000 mc. Note the blackening of the film in vascular network and lamellar bone outlining the endosteal and periosteal surfaces of the cortex. This picture suggests that bone deposition is not at a standstill and that excessive bone resorption can be responsible for osteoporosis of excessive hypercorticoidism as well as senile osteoporosis.

tory was unremarkable except for rheumatoid arthritis. There was no recognizable endocrine disturbance other than exogenous hypercortisonism.

The physical examination showed an increased posterior thoracic curve and flattening of the normal anterior lumbar curve. The patient was unable to stand or to turn himself in bed without experiencing extreme pain and discomfort in the low back and both hips. *The patient had the typical Cushinoid pale-blue thin skin* there was obesity limited to the trunk nonpitting edema of the extremities, and loss of hair The blood pressure, however was only 130/80 The laboratory findings revealed hypoproteinemia, eosinopenia, and slight hypercalcemia The serum calcium was 11.5 phosphorus 4 1 and alkaline phosphatase 5.2. X-ray examination showed wedge-shaped vertebral bodies in the dorsal spine, a pathologic fracture of the body of the first lumbar vertebra and biconcave cortical end-plates of the lumbar vertebra. The cortex of the long bones was grossly diminished in thickness and showed vertical striations of decreased bone density in the upper end of the femur equally on both sides.

On April 10 1958 patient developed auricular fibrillation and embolization at the bifurcation of the left popliteal artery Conservative treatment failed to sustain the circulation in the foot. A popliteal

exploration and embolectomy was performed the postoperative diagnosis was rheumatoid vasculitis. The circulation in the leg was measured at various intervals in the ensuing 2 weeks with the aid of several tracer doses of radioactive phosphorus P^{32} The embolus recurred, and the circulation gradually became reduced until the foot and lower leg were gangrenous. An amputation was performed 3 weeks after the embolectomy and specimens of cortical bone were removed for undecalcified sections microradiographs and automicroradiographs (Fig 2-4)

Histologic Sections. Routine microscopic preparations stained with hematoxylin and eosin showed the cortex to be one third or one half as thick as normal in the osteoporotic specimens These showed many enlarged Haversian canals filled with fatty areolar or fibrous tissue and blood vessels and almost no osteoclasts The connective tissue lining the lamellae of bone tissue however consisted of small spindle shaped cells, as in normal specimens Because typical osteoblasts are normally difficult to distinguish from ordinary connective tissue cells in adult bone tissue it was not possible on morphologic grounds to confirm the often quoted view of Pommer[13] that osteoporosis was due to low osteoblastic activity

Microradiography Undecalcified sections of cortical bone and microradiography provide information that is not otherwise obtainable [14] In osteoporosis, cortical bone showed evidence of resorption that was not apparent in samples of cancellous bone The morphologic findings suggested that the lesion in osteoporosis is principally due to *resorption* of the cortex, causing buckling and collapse of the structure of the bone. Contrary to expectations, there was more new bone formation in osteoporosis than in normal cortical bone The new bone was distinguished from the old bone by its low-density calcification Because there were no osteoid borders in corresponding areas of the histologic sections there was no possibility of osteomalacia in these cases The old bone tissue in the interior of the thin cortex was *more* densely calcified than in young nonosteoporotic individuals. The bone was resorbed and the Haversian canals were enlarged only on the endosteal surfaces of the bone, leaving the periosteal surfaces relatively unaltered Of special interest was the *hypercalcification* of the deeper lamellae of bone and the obliteration of the Haversian canals with calcium salts.

The tensile strength of a bone, as observed by Evans [15] depends upon the structure of the osteons It should be emphasized that the important lesion in osteoporosis the factor responsible for the deformities and fractures characteristic of this disorder is the loss of intact osteons

Microautoradiography Repeated tracer doses of P^{32} administered to the patient described in Case 3 showed that there was uptake of the isotope selectively by the lamellae with low-density calcification indicating that this tissue was new bone Hence it would seem that there was more new

bone formation in progress in this patient with osteoporosis than there was in control patients without osteoporosis. The condition would seem to be due primarily to bone resorption and consist, therefore of a defect in this phase of the process of Haversian remodeling

Discussion

The observations presented above are remarkable in that they constitute an extraordinary collection of negative information. Osteoporosis was not a great deal more prevalent in aged women than men Endocrine disorders were found in the minority rather than the majority of patients The disorder was present before rather than after the patient was made bedfast by a fracture of the spine or hip The dietary intake of calcium was not lower in patients with osteoporosis than in those without it. The plasma protein levels, hormonal factors such as estrogen, 17KS and 17OHCS in blood and urine and related measurable components were no different in individuals with osteoporosis than in individuals of comparable age without osteoporosis

The increased urinary calcium excretion and the development of osteoporosis following administration of adrenal steroids gave very important positive information about this disorder although the intermediate steps between intestinal absorption of corticoids and osteoporosis require more basic laboratory investigation The morphologic similarity between osteoporosis following exogenous hypercortisonism and osteoporosis occurring spontaneously in postmenopausal and senile women was apparent in triple preparations (histologic, microradiographic, and microautoradiographic) of undecalcified cross sections of cortical bone Whether these considerations have a metabolic derangement in common is not definitely known.

The literature on osteoporosis has been reviewed recently in detail [13, 18–26] and reveals that nearly all authors favor one or another of the endocrine theories of Albright and his associates The most thoroughly substantiated of these is the adrenogonadal hormone imbalance theory of Reifenstein The dietary deficiency theories have been advocated mainly by writers of years gone by although Nordin[27] has recently revived the calcium deficiency theory and argued that, with normal vitamin D intake, it created the conditions for unprotected excretion of calcium in urine and osteoporosis There are few well-documented cases in the literature to support this or the achlorhydria, or the protein starvation theories. There are overwhelmingly more cases reported to support the endocrine theory of the etiology of osteoporosis but it too is unproved and requires further basic as well as clinical investigation

The evidence against the endocrine theory is as follows (1) the majority of patients with various endocrine disorders such as ovarian insufficiency castration or senility did not develop osteoporosis[8] (2) adrenal steroids

caused fractures mainly in individuals of advanced age or those who had either osteoporosis or a disease that predisposed them to osteoporosis[27] (3) sex hormone treatment of patients with osteoporosis appeared to be nonspecific, without effect on the cause of the condition and did not restore the density of the bones (4) severe osteoporosis has been observed in a few patients who had been on a low calcium intake diet for many years (5) osteoporotic patients found in calcium equilibrium on a low calcium intake went into positive calcium balance when the calcium in the diet was increased.[28]

The evidence in support of the endocrine theory of osteoporosis is as follows (1) nearly all the "sixteen" causes of osteoporosis were endocrine disorders (2) one endocrine mechanism involving the metabolism of adrenal glucocorticoids could have been common to all causes (3) prolonged administration of cortisone and related adrenal cortical steroids produced osteoporosis resembling osteoporosis of Cushing s syndrome in man[29] (4) administration of sex hormones to patients with postmenopausal senile, or hypercorticoid osteoporosis produced calcium and nitrogen retention and relieved the patient of further fractures (5) administration of sex hormones produced positive calcium and nitrogen balance in men with osteoporosis but not in men of comparable age without osteoporosis[30] (6) the hormonal metabolite, present or absent, responsible for osteoporosis is not yet known and need not necessarily be revealed by measuring hormones free bound, or conjugated in the plasma and urine in chronic disorders the active form of most steroid hormones is in a steady state and blood levels do not disclose the level of production or utilization (turnover) The literature following the works of Albright and his associates lists 18 causes of osteoporosis. Urist suggested that there could be one mechanism common to all causes, and this is the resorbing action of normal levels of 17OHCS production upon cortical bone Hypertrophy of the *adrenal cortex was found in birds on calcium-deficient diets.*[31] Protein deficient diets and starvation have also been found to produce similar adrenal cortical overactivity Thus the effects of dietary deficiency would not be separable from the effects of hormonal especially adrenal regulation of calcium and bone metabolism.

Aging, Individual Genetic Factors, and Susceptibility The hereditary aspects of osteoporosis seem not to have been investigated It has been suggested that one individual s constitutional make up may predispose him to osteoporosis,[19] while another individual may develop diabetes, hypertension, or gastric ulcers or some other concomitant of aging Osteoporosis often develops so slowly over a period of so many years that aged individuals forget that they had back pain and assume that to become bent over and shorter in stature is a natural infirmity of growing old. There is no reason to assume however that osteoporosis is a manifestation of aging

The author has made x ray examinations of the spine of several women

individual against high or normal levels of 17OHCS production This may be present in the zona reticularis of the adrenal cortex the liver the muscle or the bone tissue itself

Summary and Conclusions

1 The principal etiologic factor in the pathogenesis of osteoporosis irrespective of interrelationships with menopause senility, endocrinopathy nutritional deficiency pregnancy etc., is not known Osteoporosis is usually asymptomatic in aged men but is almost as common as in aged women. There is no correlation between dietary intake of calcium and osteoporosis

2. Approximately 9 per cent of the nonosteoporotic men and 18 per cent of the osteoporotic men were under treatment for gastrointestinal tract ulcers there were no ulcers in the nonosteoporotic women but 4 per cent of the osteoporotic women had ulcers To investigate such neuroendocrine interrelations it seems necessary to search further for nonsymptomatic ulcers in patients with osteoporosis.

3 Specific hormone disorders, hypogonadism diabetes hyperthyroidism, were slightly more common in women than in men with osteoporosis, but the great majority of patients with osteoporosis had no specific endocrine disease

4 Except in patients with endogenous or exogenous hyperadrenocorticoidism, osteoporosis develops at an apparently normal level of 17OHCS production.

5 The lower level of the plasma albumin and the lower level of the urinary 17KS and estrogens (in the presence of relatively normal level of the 17OHCS) were generally the same in nonosteoporotic subjects as in patients with osteoporosis The level of the 24-hour urinary calcium excretion however was almost invariably low in patients with severe osteoporosis It was not possible to distinguish individuals with osteoporosis from individuals without osteoporosis by means of the ACTH tolerance cortisol metabolism, and prednisone urinary calcium excretion tests

6 Adrenal biopsies in one case of senile osteoporosis showed normal zona fasciculata but atrophy of the other layers

7 Triple preparations of undecalcified cortical bone examined in cross section by combined histologic, microradiographic and microautoradiographic techniques, show that in osteoporosis the bone resorption phase of Haversian remodeling appeared to have greatly accelerated and that the bone reconstruction phase was active but not sufficient to replace the loss of bone mass Bone resorption in osteoporosis occurs almost entirely without the action of osteoclasts

8 Osteoporosis of the spine and hips is becoming increasingly common in the census of all orthopedic services and should receive a proportionately greater amount of attention from orthopedic surgeons This disorder is

responsible for the unalterable 30 per cent incidence of nonunion in fractures of the hip not because of inability of the bones to heal but because osteoporosis causes crumbling fracture surfaces For this reason it was technically impossible to achieve good contact and internal fixation by metallic appliances in many cases An examination of lateral view films of the spine is recommended in order to determine the presence or absence of osteoporosis and avoid ineffectual methods of metallic internal fixation in many patients with fractured hips.

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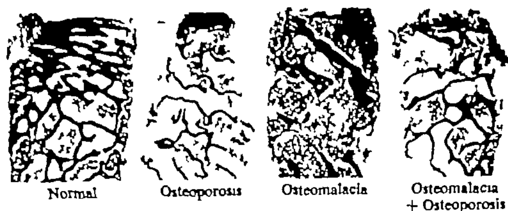


FIG 3 2. Iliac crest biopsies of normal, osteoporotic, osteomalacic, plus osteoporotic bone. (×15)

sponds to the first block in Fig 3-1 the osteoporosis biopsy with the thin trabeculae to the second the osteomalacia biopsy to the third, and the biopsy showing both osteomalacia and osteoporosis to the fourth Figure

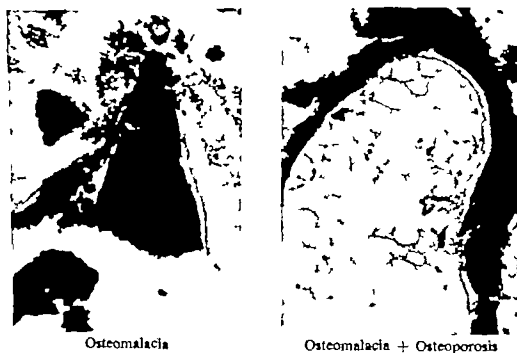


FIG 3 3 High-power magnification of undecalcified sections from the last two samples in Fig 3 2 to show osteoid borders. (×150)

3-3 shows the osteoid borders in undecalcified sections of the last two specimens *

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* Full details of the assessment of osteoporosis in iliac crest biopsies will be published shortly in collaboration with Dr J Beck Department of Pathology Western Infirmary

3

Osteoporosis and Calcium Deficiency

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The Definition of Osteoporosis

Osteoporosis is a condition in which there is a reduction in bone mass without any reduction in the ash content of the bone or any other known change in its structure or composition The simple diagrams in Fig 3-1 illustrate this concept. If the large block represents the normal skeleton, then the smaller one at its side with the same ash content, represents an osteoporotic skeleton The third block represents a case of rickets or osteomalacia, in which the skeletal mass is normal, but its ash content is greatly reduced. Finally in the fourth block there is the important combination of osteomalacia and osteoporosis which Atkinson, Nordin, and Sherlock[1] have shown in steatorrhea and which is of great practical importance as well as theoretical interest.

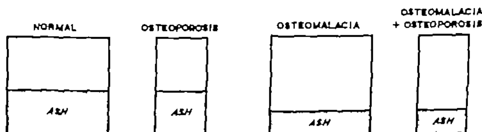


FIG. 3 1 Diagrammatic representation of the principal metabolic abnormalities of the skeleton.

The four blocks in Fig 3 1 have their counterpart in the histology of the skeleton, as shown in Fig 3 2 The normal iliac crest biopsy corre-

per day I have collected a further 92 balances on normal subjects of all ages[12, 13] I have excluded from the data those cases in which the authors stated that osteoporosis was present These balances are shown in Fig 3-4

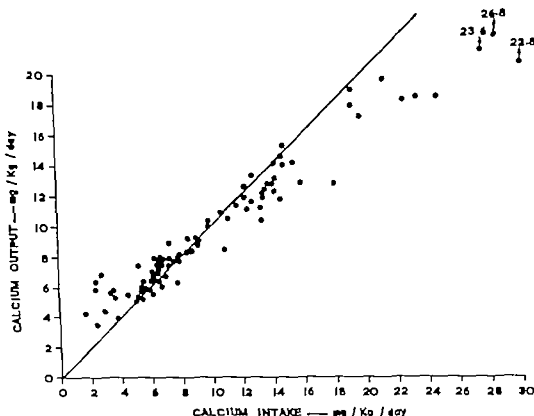


FIG 3-4 Calcium balances in 92 normal individuals.

The regression of output on intake is represented by the formula $y = 0\ 735x + 2\ 3$ and the 95 per cent confidence limits are $\pm\ 2\ 1$ mg/kg

The mean requirement is thus about 10 ± 2 7 mg/kg/day However the individual requirements in 40 balances on 32 of these normal subjects in which it has been possible to calculate them are shown in Fig 3-5 The striking feature is that the requirement ranges up to 19 mg/kg/day

Dietary surveys suggest that the *average* intake of calcium in Great Britain and the United States is in excess of the average requirement. This is far from meaning however that the whole population is necessarily on an adequate intake Thus a dietary survey of 303 old people in Britain by Bransby and Osborne[16] revealed that 19 of them were taking less than 400 mg of calcium daily Unless they were all very small and light, this would represent an intake well below the average requirement defined by balance studies Clark[17] found that 23 per cent of an American population sample running into several thousands were taking less than 75 per cent of the recommended intake of calcium though it must be admitted that the American recommended intake is high (1 Gm daily)

Historic Background

The distinction between osteoporosis and rickets was first laid down by Pommer[2] in 1885 in a classic monograph He showed that the important difference lay in the presence of the osteoid border in rachitic bone in osteoporotic bone the trabeculae were thin but had no excessive osteoid seams Pommer suggested that osteoporosis was due to impaired formation of new bone, but a number of other German workers, Miva and Stoeltzner[3] and Reimer and Boye,[4] soon showed that osteoporosis could be produced in dogs by low calcium diets and they concluded that the condition was therefore due to bone destruction. They also inferred that rickets must be due to some factor other than calcium deficiency

What this other factor might be was not established until after the First World War when Mellanby[5] showed that it was a fat-soluble vitamin which soon came to be known as vitamin D It was not long before Telfer[6] and other workers showed that there was malabsorption of calcium in rickets and that this could be corrected by administration of vitamin D German medicine had by this time lost the leading position it had previously held, the *prewar German work on calcium deficiency and osteoporosis had been for* gotten and it was apparently assumed that the malabsorption of calcium in rickets was the cause of the pathologic lesion From this it was inferred that calcium deficiency must also produce rickets and this is still implied in contemporary texts.[7]

It must have been in this climate of opinion that Albright, some time in the 1930s realized that the common spinal rarefaction of the middle aged and the elderly was not a form of rickets but was in fact due to osteoporosis [8] Believing as he did that calcium deficiency must produce osteomalacia,[9] he postulated that osteoporosis was due to a reduction in the formation of bone matrix [10] a hypothesis which is still widely accepted. It is possible to argue however that vitamin D deficiency and calcium deficiency are different conditions and that osteoporosis might be due to the latter

The Possibility of Calcium Deficiency in Man

Is there such a thing as dietary deficiency of calcium? To answer this question one needs to know what the individual calcium requirement is and how the actual intake compares with this requirement One must then consider whether human beings can adapt to what would otherwise be inadequate intakes

Balance data provide the only real clue to the calcium requirement of human subjects. In 1939 Mitchell and Curzon[11] published a summary of 139 balance studies on normal subjects collected from the literature These suggested that the average requirement was about 10 mg/kg body weight

FEMUR The total thickness of the cortex at its thickest point in the PA view compared with the total diameter at the same point (B)

HAND The total thickness of the cortex of the second metacarpal of the right hand at its thickest point compared with the total diameter (C)

Table 3-1 below presents the criteria on which cases have been included in this series.

Radiologic Definition of Osteoporosis

Central—Biconcavity ($A < 0.81$) or crush fracture
Peripheral—Abnormal hand and femur (B and $C < 0.50$) with normal spine
Mixed—Abnormal A, B and C

Ninety two cases have been collected which are abnormal by these criteria of these, 31 are central, 30 are peripheral and 31 are mixed Only cases of what I would like to call primary osteoporosis have been included in the series Thus patients with hyperthyroidism, Cushing's syndrome, or other metabolic disorders known to affect bone by unexplained mechanisms have been deliberately excluded The cases are such as other workers would call "idiopathic," "postmenopausal" or "senile" osteoporosis but which I prefer to call primary osteoporosis The present communication must not be taken to imply that I necessarily believe secondary osteoporosis to be due to negative calcium balance

Table 3-1

POSSIBLE PATHOGENESIS IN 92 CASES OF OSTEOPOROSIS

Pathogenesis	*Type of osteoporosis*			*All cases*
	Central	*Peripheral*	*Mixed*	
Steatorrhea	4	0	6	10
Low intake (<14 mg/kg)	17	16	15	48
Hypercalciuria (Ca/Cr > 0.28)	7	3	4	14
Remainder	3	11	6	20
Total	31	30	31	92

Dietary Intake of Calcium. With the help of Miss McCombie the Chief Dietitian of the Western Infirmary and her staff diet histories have been obtained from all these patients and from an equal number of control subjects without backache selected from the hospital population, with a bias toward elderly women. The comparability of the two groups in terms of age and sex is shown in Fig 3-6 There are rather more men in the control group and the average age of the controls is a little less than that of the patients with osteoporosis. Neither of these small differences can explain the difference in dietary intake between the two groups

However Hegsted et al [18] would argue that human beings can adapt to low calcium intakes over long periods and that there is therefore no such thing as a calcium requirement. It is true that evidence of adaptation has been produced by Walker et al.[19] and Malm et al.[12] but the fact that *some*

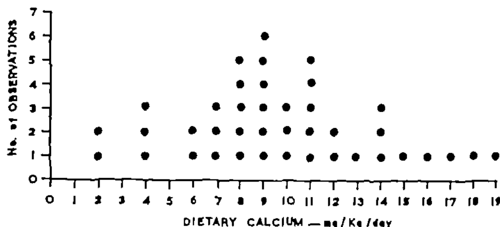


FIG 3-5 Individual calcium requirements calculated from data in Fig 3-4

people can adapt does not prove that *everyone* can do so In fact, if one examines Malm's data carefully one finds that some of his subjects did not adapt to these diets but remained in negative balance for months on end. Is it not possible that the people who cannot adapt are the very ones who develop osteoporosis if their diet is inadequate?

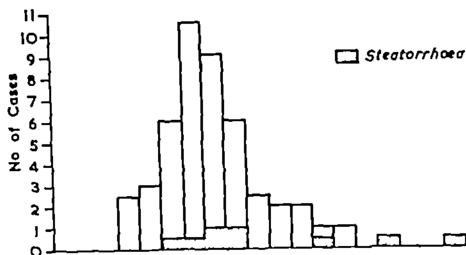
Observations in Clinical Osteoporosis

For the last 2 years, I have been collecting data on patients with osteoporosis in order to discover whether or not their condition might be due to prolonged negative calcium balance, and I can now report some preliminary results Negative calcium balance could of course be produced in three different ways—dietary lack of calcium malabsorption, or hypercalcinuria—and these three factors will be considered in turn it is of course possible for more than one of them to be operative in any given case

Radiologic Definition of Osteoporosis. The cases have been defined radiologically No attempt has been made to assess bone density Instead, we have made the following standard measurements on the bone x rays and compared them with similar measurements in control films of normal subjects of all ages and both sexes This work is being done in conjunction with Dr E. Barnett of the Department of Radiology and is not yet complete, but provisional normal standards have been established. The measurements are as follows.

SPINE The vertical height of the best centered lumbar vertebra at its narrowest point compared with the vertical height at the anterior margin (*A*)

OSTEOPOROSIS



CONTROLS

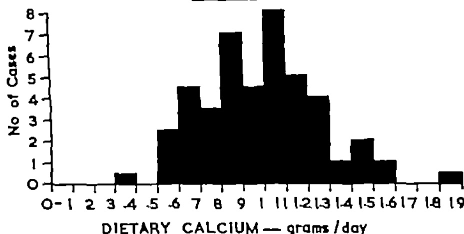


FIG. 3-7 Dietary calcium intake in 92 osteoporotic patients and 92 controls.

ent. Four of these patients were known to have steatorrhea before their bones were x rayed 6 of them were known to have osteoporosis before their fecal fat was estimated. The fecal fat was normal in 23 more cases In the remainder it has not yet been measured It must be emphasized that biochemically and histologically these patients did not have osteomalacia.

Urinary Excretion of Calcium. In work which is being published elsewhere, I have drawn attention to the relatively small effect of dietary calcium intake upon urinary excretion. Figure 3-11 shows the urinary calcium in relation to dietary calcium in the 92 balances on normal subjects collected from the literature referred to above Figure 3 12 shows similar data from 48 balance periods in 22 of our 92 osteoporotic patients In both figures it is clear that there is no relationship between dietary and

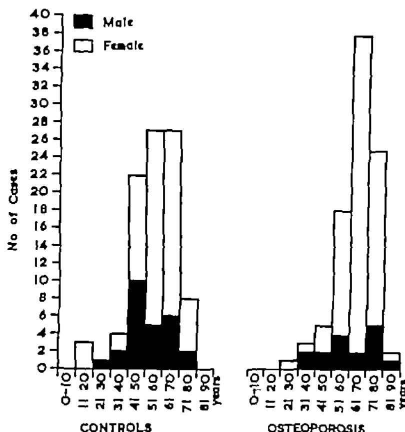
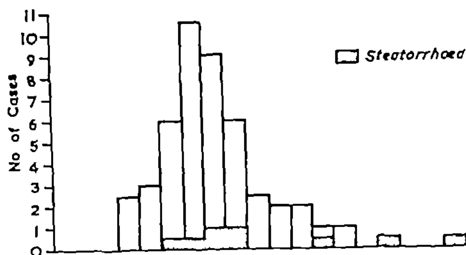


FIG. 3-6 Age and sex distribution in 92 osteoporotic patients and 92 controls.

Figures 3-7 and 3-8 show the dietary intake of calcium in the two groups It will be seen that, whereas only 19 of the controls have dietary intakes of less than 14 mg/kg body weight, 50 of the osteoporotic patients have intakes below this level and 7 of those with higher intakes have steatorrhea. The difference between these two groups (excluding all cases of steatorrhea in which malabsorption is believed to be the operative factor) is significant at the 1 per cent level Figure 3-9 shows that there is also some difference in the protein intake of the two groups probably due to the lower milk intake of the osteoporotic patients The relationship between calcium and protein intake is shown in Fig 3 10 When calculated on a body weight basis, the difference between the protein intakes of the two groups is significant at the 5 per cent level but none of my patients so far has been in negative nitrogen balance on their home intake of protein.

Malabsorption of Calcium. Ten of the patients had steatorrhea, i.e., they excreted more than 5 Gm of fat daily in the feces over a 4- or 8-day period and it seems reasonable to assume that malabsorption of calcium was pres-



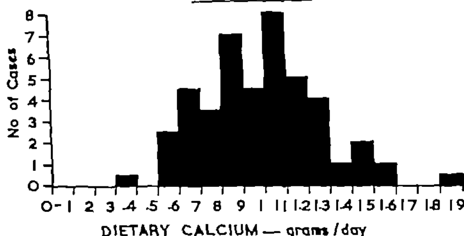


FIG 3-7 Dietary calcium intake in 92 osteoporotic patients and 92 controls

ent Four of these patients were known to have steatorrhea before their bones were x rayed 6 of them were known to have osteoporosis before their fecal fat was estimated The fecal fat was normal in 23 more cases In the remainder it has not yet been measured It must be emphasized that biochemically and histologically these patients did not have osteomalacia

Urinary Excretion of Calcium. In work which is being published else where, I have drawn attention to the relatively small effect of dietary cal cium intake upon urinary excretion Figure 3-11 shows the urinary calcium in relation to dietary calcium in the 92 balances on normal subjects col lected from the literature referred to above Figure 3-12 shows similar data from 48 balance periods in 22 of our 92 osteoporotic patients In both figures it is clear that there is no relationship between dietary and

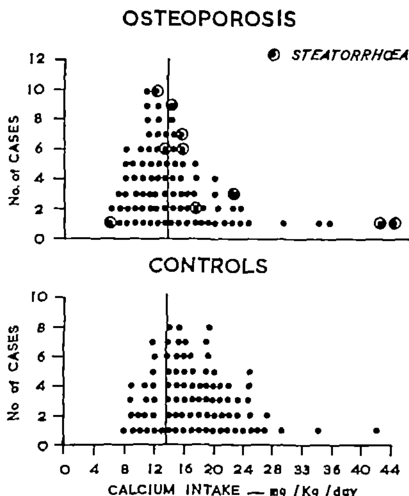


FIG 3-8 Dietary calcium intake per kilogram in 92 osteoporotic patients and 92 controls.

urinary calcium. This does not mean that some small reduction in urine calcium cannot be obtained by a low calcium diet but simply that the variations in urine calcium between one individual and another are greater *than any changes due to diet. This has previously been noted by Nicolaysen* et al [39] and Bogdonoff et al [15]

This feature of urine calcium excretion has at least two implications. The first is that it should not normally be necessary to put patients on a low calcium diet before measuring their calcium output, although it is true that in certain circumstances and for certain purposes this may be a useful procedure The second is that it may help to explain why osteoporosis develops on low calcium diets—if the urinary excretion of calcium is relatively fixed in any given individual, then clearly he or she cannot remain in positive balance on a low intake

No satisfactory standards exist for the assessment of urinary calcium

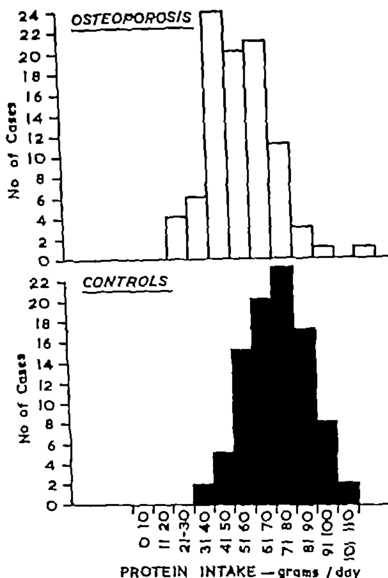


FIG. 3 9 Dietary intake of protein in 92 osteoporotic patients and 92 controls. (Compare with Fig. 3 7)

output We have found that there are considerable variations between one individual and another and even in one individual from day to day on a fixed diet, but that these variations can be greatly reduced by expressing calcium output in relation to creatinine This calcium/creatinine ratio or Ca/Cr (each estimation being expressed in milligrams per 100 cc) has been found to range from 0 03 to 0 28 in random samples of urine from 71 normal subjects on their own home diet or on a ward diet, only persons on a high milk diet being excluded The 24-hour calcium creatinine ratio is rather narrower but has not yet been finally defined.

The calcium/creatinine ratio in a random sample of urine in the 92 cases of osteoporosis is shown in Fig 3-13 in relation to the dietary intake of

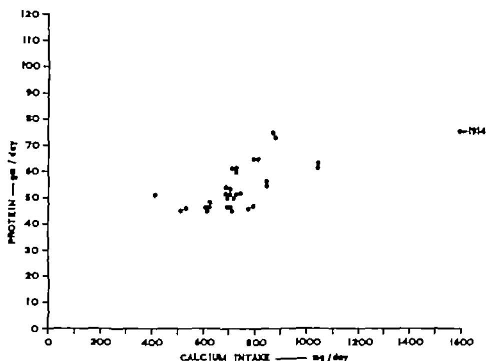


Fig. 3 10. The relationship between calcium and protein intake m osteoporosis.

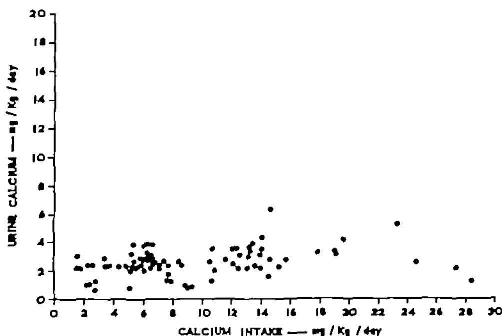


Fig. 3 11 Urine calcrum and dretary calcium in 92 normal subjects collected from the literature.

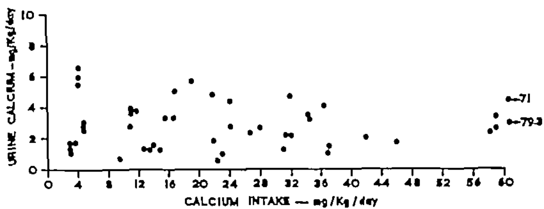


FIG. 3-12. Urine calcium in osteoporosis.

calcium as obtained from the diet history It will be seen that 26 of the 92 cases have some degree of hypercalcinuria by this definition, these high values being of the same order as those observed in a series of patients with hyperthyroidism which is being reported elsewhere Eleven of the cases have low calcium intake and high rates of excretion

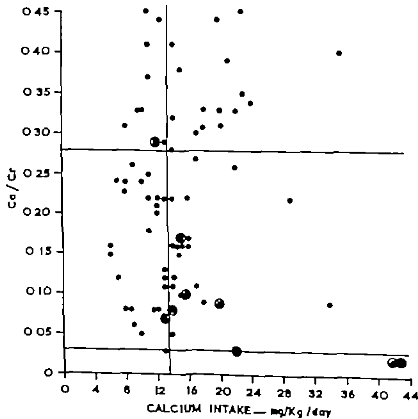


FIG 3-13 Dietary calcium and urinary calcium in 92 cases of osteoporosis

In Fig. 3-14 the osteoporotics and controls have been combined to show the relationship between osteoporosis and dietary intake of calcium and urinary excretion. In the right histogram, there are 29 subjects with intakes below 10 mg/kg/day about three-quarters of these had osteoporosis (shown in black) There are 35 subjects with intakes above 20 mg/kg/day

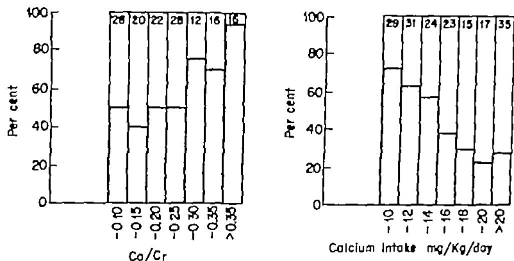


FIG. 3 14 Dietary calcium and urine calcium in osteoporosis and control subjects. Vertical scale shows percentage of osteoporosis (in black) in each group Figures at top of columns signify total number of subjects.

and only about one-quarter of them had osteoporosis. Conversely the left histogram shows that only about half the subjects with low urine calcium/creatinine ratios had osteoporosis, whereas 95 per cent of the 16 subjects with ratios above 0.35 were suffering from osteoporosis Thus osteoporotic individuals tend to have lower intakes and higher urinary excretion of calcium than normal subjects and I suggest that this is why they develop the disease (It should be noted that cases of steatorrhea have been excluded from these histograms and that it was not possible to get urinary Ca/Cr figures from all the controls)

Possible Pathogenesis of Osteoporosis. Table 3 1 summarizes the results in relation to intake, absorption and excretion of calcium In 10 of the patients, the presence of steatorrhea suggests that malabsorption of calcium was the cause of the osteoporosis. In 48 of the remainder the dietary intake was less than 14 mg of calcium per kilogram a level of intake which was recorded in only 19 of the 92 controls Finally a calcium/creatinine ratio in excess of 0 28 was recorded in 14 cases. Thus malabsorption low intake or high output could explain negative calcium balance in 72 of these 92 cases There remain 20 cases which it is not possible on the available data to explain on these lines. It may or may not be significant that only 3 of these cases are in the central group and that 2 of them have been shown by

balance studies to be in negative balance because of malabsorption though having no steatorrhea One patient has since responded to small doses of vitamin D, and the other was suffering from chronic diarrhea without steatorrhea and went into positive balance when the diarrhea was controlled On the other hand there are 11 unexplained cases in the peripheral group

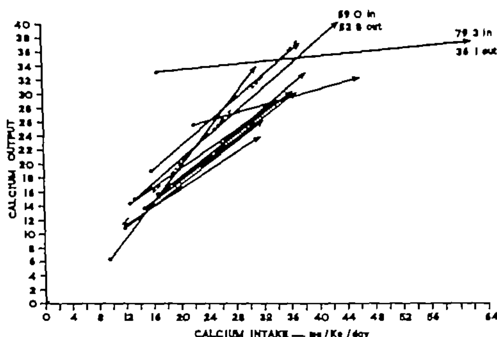


FIG 3 15 Calcium balances in osteoporosis Low intakes represent patients own home diet.

Balance Studies. The results of 22 balance studies in 11 cases are shown in Fig 3-15 All diets were analyzed. All collections were preceded by an equilibration period of at least 4 days at each level of intake and the collection periods ranged from 8 to 28 days The lower intake level represents the closest possible approximation to the patient s own home diet, and the higher one was obtained by giving supplements usually in the form of calcium glycerophosphate Of the patients 6 were in negative calcium balance on their home intake and 5 in positive balance When supplements were given substantial positive balances were achieved in 8 of the patients but not in 3 Two of these three were of the peripheral type The third was subsequently given 1 000 units of calciferol daily and a larger dose of calcium as an outpatient. A year later when she was symptom-free she was readmitted for a further balance, as shown in Fig 3 15 (case M G) and she was then found to be in considerable positive balance

It may be argued that the positive calcium balance which can be achieved in acute observations cannot be maintained. We have therefore performed

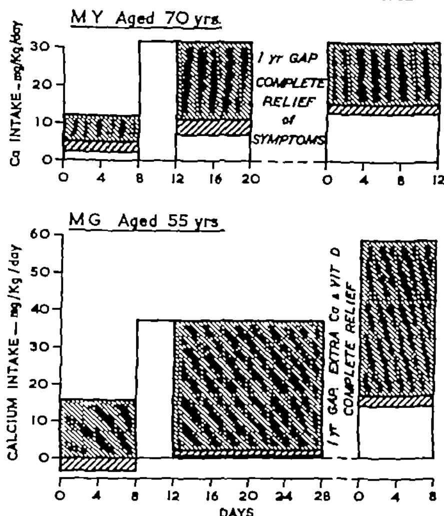


FIG. 3-16. Calcium balances in osteoporosis. (Note positive balances shown by space above base line, negative balance below base line.)

four balance studies in patients who have been on a high intake for a year or more *The results are seen in Figs. 3-16 and 3-17 which show that all* these four cases were in strongly positive balance even after this long period on a high intake

Calcium Therapy Patients with backache are being treated with oral calcium supplements, usually in the form of calcium glycerophosphate Gm 2 or 4 three times daily The effect on the backache in 47 patients who have been observed for at least 3 months is shown in Table 3-2. Back ache was wholly or largely relieved in the great majority of these patients It is of course notoriously difficult to assess the significance of symptomatic relief in any condition but it seems unlikely that this response was a placebo effect both because of the high proportion of positive results and because it did not occur immediately but usually appeared within about

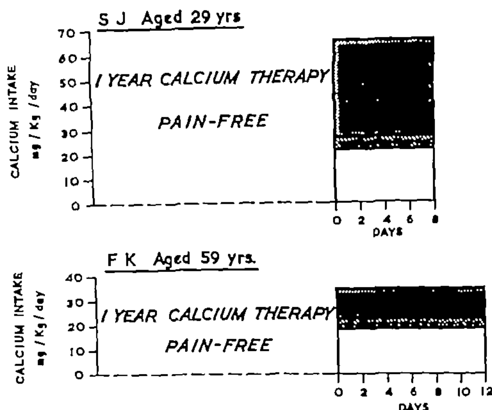


FIG. 3 17 Calcium balances in osteoporosis

2 to 3 months after starting treatment. Furthermore there does appear to be a *relationship in a small number of cases between the results of balance* studies and the relief of symptoms Thus all the patients shown in Fig 3-15

Table 3-2

RESPONSE OF PAIN TO CALCIUM THERAPY 47 CASES

Response	*No*	*Per cent*
Pain-free	15	
Very much better	14	79
Improving	8	
Improved after vitamin D	3	6
No change	7	15

who went into satisfactory positive calcium balance have been relieved of pain whereas two of those who did not are still complaining of backache The third patient who failed to achieve a positive balance was M G shown in Fig 3 14 and already referred to who continued to complain of severe pain until she was given vitamin D 1 000 units daily and the dose of calcium was doubled.

Accretion Rate. Last but by no means least I come to the measurement of bone accretion rate with radioactive calcium by the method of Bauer et al.[31] *We have done this with calcium[47] in a small number of cases*, and the results are shown in Table 3-3 If the Albright hypothesis were correct that

Table 3-3

RADIOCALCIUM STUDIES IN 4 NORMAL SUBJECTS AND 8 PATIENTS WITH OSTEOPOROSIS

Weight *kg*	*Exogenous* Ca, *mg*	*Exogenous* Ca/*kg* *body weight* *mg/kg*	*Endogenous fecal* Ca, *mg/day* *(average)*	*Accretion rate*	
				mg/day	*mg/day/kg*
			Normal		
65	7,540	116	200	870	14.5
40	4,500	92	180	560	11.6
72	2,970	41	140	550	7.6
64	2,080	47	170	700	10.9
			Osteoporosis		
60	3,290	54	80	650	10.9
60	3,060	51	100	460	7.6
50	3,890	78	220	520	8.5
34	2,800	83	80	610	17.7
34	3,040	107	90	600	16.3
45	2,020	65	170	680	15.0
54	2,460	46	70	410	7.6
74	3,900	53	170	810	11.2

this is a disorder involving impairment of new bone formation, then one would of course expect the accretion rate as measured by this technique to be low However as the table shows, the accretion rate is much the same in the 8 cases of osteoporosis as in the 4 normal subjects whom we have tested so far I understand that other workers are obtaining similar results.

An Explanation and a Hypothesis

The suggestion that osteoporosis may be caused by calcium deficiency is supported by the extensive literature on low calcium diets in animals which there is not time to mention on this occasion, but which I am reviewing fully elsewhere. This work and the results which I have reported above indicate in my opinion that vitamin D deficiency and calcium deficiency produce two different conditions in the latter plasma calcium and phosphate levels are normal but the skeleton is slowly destroyed by the negative balance whereas in the former the calcium × phosphate product is low and new bone does not calcify Vitamin D deficiency must there

fore have some effect upon plasma calcium which is independent of or additional to its effect upon calcium absorption This action of vitamin D has in fact been demonstrated by Carlsson and Lindquist [73] who found that increasing doses of vitamin D given to different groups of rachitic rats progressively raised the plasma calcium level in a manner that could not be explained by any effect upon calcium absorption In other words vitamin D has some direct action upon bone which has the effect of raising plasma calcium It is this action which is presumably responsible for the hypercalcemia and bone destruction of vitamin D intoxication

If this action of vitamin D is accepted, it is possible to explain the difference between osteomalacia and osteoporosis in terms of the different effects of vitamin D and calcium deficiency respectively on the plasma calcium level The essential feature of this concept is the state of dynamic equilibrium which must exist between the mineral in the skeleton and that circulating in the extracellular fluid. In normal circumstances, this equilibrium is set at such a level that the plasma calcium is about 10 mg per 100 cc, but at least two factors are required for the maintenance of this level—parathyroid hormone and vitamin D In the absence of the parathyroids, the plasma calcium drops to a lower level but can be raised again by the administration of large doses of vitamin D In vitamin D deficiency the plasma calcium also drops but this stimulates the parathyroids which respond by maintaining the plasma calcium at a near normal level at the same time they lower the plasma phosphate and so produce the low calcium × phosphate product which leads to rickets

Figure 3 18 is an attempt to explain this situation in diagrammatic form The skeleton is represented as a large reservoir of fluid (denoting calcium) which is in equilibrium with a small tank representing the extracellular fluid. The level of fluid in the latter is governed by the level in the reservoir and the level of the whole reservoir is sustained by the combined actions of vitamin D and parathyroid hormone, represented by supports underneath it. Removal of either of these blocks leads to an appreciable fall in the calcium concentration in the extracellular fluid but this can be restored by excessive activity or administration of the other principle

In negative calcium balance without vitamin D or parathyroid deficiency whether this is due to inadequate intake malabsorption, or relative or absolute hypercalcinuria the level of fluid in the smaller tank will be maintained by the fluid from the larger tank and if the larger tank is much greater than the smaller one no appreciable change in the fluid level will occur at least for a very long time In other words if normal vitamin D and parathyroid activity have set the equilibrium at a plasma calcium of 10 mg per 100 cc, the skeleton may be expected to sustain this level by giving up mineral (and subsequently losing matrix) *without any additional intervention from the parathyroid glands*

If this hypothesis is correct, neither low calcium diets nor hypercalcinuria

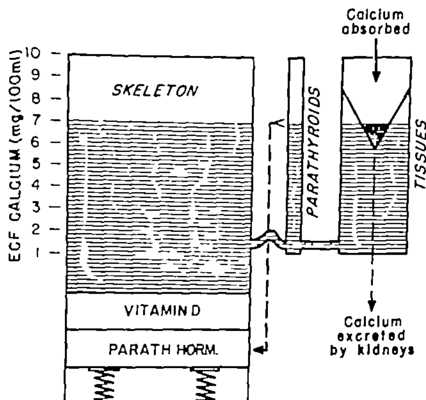


FIG. 3 18. Diagrammatic representation of calcium equilibria to indicate how serum calcium is sustained by mineral in large skeletal reservoir The extracellular fluid calcium level is assumed to be 70 per cent of the serum calcium.

should have any effect upon plasma calcium concentration or parathyroid activity if the skeleton itself is fully mineralized. This conflicts with the popular view of Albright and Reifenstein[9] and Pines and Mudge[23] that the hypercalcinuria of renal tubular acidosis leads to parathyroid stimulation and osteomalacia, but in this condition the acidosis itself is an additional factor which may explain the picture In simple hypercalcinuria without acidosis (which is quite a common condition) osteomalacia does not occur nor is there any lowering of the plasma calcium level. The same is true of low calcium diets at least in short term observations. If para thyroid intervention were required to sustain the plasma calcium level in the face of calcium deficiency one would expect some secondary fall in plasma phosphate on low calcium diets. I have looked for this but failed to find it.*

* Since this paper was given, the x ray standards have been more sharply defined and we now regard a combined hand and femur score of less than .89 as abnormal There are therefore a few cases of "peripheral osteoporosis" in this series which we no longer regard as abnormal. This does not affect the significance of the results as reported. In speaking of vitamin D deficiency as the cause of osteomalacia, the author envisages a severe degree of vitamin deficiency It is possible that mild vitamin D deficiency causing only malabsorption of calcium would lead to osteoporosis, and this may be why osteoporosis is seen in some cases of steatorrhea.

Summary and Conclusions

I am suggesting that osteoporosis is the result of negative calcium balance rather than the cause My argument rests upon the following points

1 Calcium deficiency produces osteoporosis in animals

2 In the majority of a series of patients with clinical osteoporosis evidence was obtained of low calcium intake malabsorption or hypercalcinuria.

3 The calcium accretion rate in a small number of patients was normal

4 Most patients with osteoporosis can be put into positive balance simply by feeding calcium This positive balance appears to be sustained

5 In the great majority of patients, backache can be relieved or cured simply by feeding calcium supplements

6 There are theoretical grounds for believing that calcium deficiency would lead to osteoporosis.

7 It is known that some individuals in our society are eating very little calcium, and it is very possible that a proportion of these are in negative calcium balance

8 It is inherently probable that, since 99 per cent of body calcium is in the skeleton a reduction in skeletal mass would be due to mineral rather than protein deficiency This hypothesis has the added advantage of simplicity

I should like to emphasize again that these results of mine are preliminary and will appear elsewhere in more detail

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4

Osteoporosis Atrophy of Disuse

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The role of atrophy of disuse in the development of osteoporosis has been a relatively noncontroversial subject. It seems generally accepted that mechanical stresses and strains logically stimulate processes of bone formation and calcium deposition In the absence of these physical impulses, *present theory has it that osteoblastic activity is reduced so that, resorption* continuing at a normal rate, less and less net bone is present and osteoporosis develops. One cannot help wondering however whether this straightforward and simple concept will always remain so as new clinical information and experimental data are inevitably acquired.

Historically probably the first detailed experimental study of the action of disuse on bone was made by Allison and Brooks[1] in 1921 In dogs with forelimb immobilization by brachial plexus section, excision of the upper end of the humerus or fixation in plaster bone atrophy seemed to be proportional to the degree of nonuse these investigators stated that immobilized bone was thinner lighter and had diminished breaking strength as the result of less bone substance without change in chemical composition. The first experimental study of inactivity in human beings was made by Cuthbertson[2] in 1929 Only in one subject, however was urinary calcium measured both before and during the recumbency phase of 11 days urinary calcium apparently did increase during that short period

During the early 1940s Armstrong, Knowlton and Gouze[3] produced bone rarefaction in rats by brachial plexus section for the purpose of observing the effects of gonadal hormones they found that estrogen tended to prevent bone atrophy whereas testosterone did not Also at this time there began to be much more clinical awareness of the possible effects of immobilization or disuse on bone as evidenced particularly by two case reports from Albright and his associates In one of these [4] a 14-year-old

boy who fractured his femur through a solitary bone cyst developed such marked hypercalcemia and hypercalcinuria that his neck was explored, unsuccessfully for a parathyroid tumor before it was realized that immobilization might be the principal cause for the continuing difficulty: the boy was mobilized with rapid improvement The other clinical experience of note[3] was the immobilization of a 64-year-old woman with Paget's disease and a fracture of the femur in this case immobilization in plaster resulted in a fall in serum alkaline phosphatase as well as hypercalcemia.

These findings in immobilized patients were reported at a time when problems of convalescence and rehabilitation were assuming great importance for the Second World War was in progress Various project areas in the study of convalescence were then organized under the Office of Scientific Research and Development one of these was to determine the effects of bed rest or immobilization and, by studying normal subjects, to differentiate the effects of immobilization per se from those which might be superimposed by disease The Keys-Minnesota study[4, 7] emphasized physiologic changes and did not measure calcium excretion. The Cornell–Russell Sage study of Deitrick, Whedon and Shorr[8] contributed metabolic data on four healthy young men who were immobilized in plaster casts from

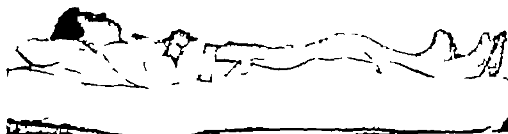


FIG 4-1 Normal healthy man, aged 21 years In bivalved plaster cast. Subjects remained in casts constantly during immobilization phase (average 23¼ hours daily) except for use of bedpan and certain physiologic tests. (*Reproduced by permission of American Journal of Medicine*)

waist to toes for 6 to 7 weeks (Fig 4-1) Including the several weeks pre- and postcontrol phases of the study the subjects were on constant dietary intakes and collections of all urine and stool for 5 months

The principal findings of this study of interest to this discussion were that immobilization brought about a prompt and gradually increasing calcium excretion, both urinary and fecal, which became maximal at 4 to 5 weeks (Fig 4-2) This pattern of gradually increasing urinary excretion of calcium to a plateau was virtually identical with that seen by Howard[9] in immobilized fracture patients quantitatively the urinary calcium increase in the normal subjects was between twofold and threefold, and the mean maximum for the group of 342 mg/day was approximately two-thirds that of Howard's fracture group The accumulated negative calcium

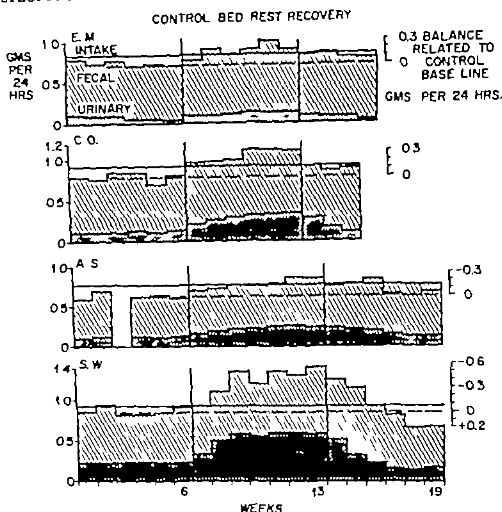


FIG. 4-2. Effect of immobilization on the calcium metabolism of four normal men. In each subject the daily calcium intake was kept constant throughout all periods of the experiment. For each subject the control base line (interrupted horizontal line) is an average of the total outputs of the last four control weeks. (*Reproduced by permission of Medical Clinics of North America*)

balance averaged 14 Gm but osteoporosis was not noticeable by x ray at the end of the 6 to 7 weeks immobilization phase Of pertinence to the high incidence of renal tract calculus formation in immobilized patients[14-16] was the observation that urinary citric acid, previously noted in ambulatory individuals to vary with urinary calcium output,[17] failed to rise in this study with the elevation of urinary calcium Urinary phosphorus also increased, and urinary pH rose slightly so that conditions in the urine became less suitable for calcium phosphate solubility Other points which are perhaps important to mention are that serum calcium tended to rise (mean increase 0 8 mg per 100 cc) that the increase in urinary nitrogen followed a different pattern from calcium, tending to peak during the second week of bed rest, and that no consistent changes occurred in urinary 17 ketosteroids or corticoids

The next phase of these studies was concerned with measures which might be applied to counteract the apparent disadvantageous effects of long immobilization. It seemed self-evident that any patient in whom it was safe should be mobilized within approximately 3 weeks of onset of disease but having in mind patients with severe orthopedic injuries or marked paralysis on a vascular or neuromuscular basis, experimental study[18] was made of a method of applying some degree of weight-bearing and increased

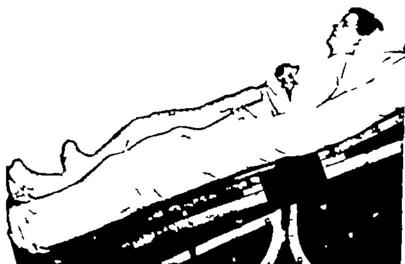


FIG 4-3 Normal healthy young man in bivalved plaster cast on Sanders slowly oscillating bed. Two minutes are required for bed to rock from horizontal to 20 foot-down position shown and back to horizontal. (*Reproduced by permission of American Journal of Medicine*)

peripheral circulation during confinement to bed (Fig. 4-3) The Sanders slowly oscillating bed,[19] used primarily in the treatment of peripheral occlusive arterial disease was employed in an immobilization study which was slightly shorter but otherwise identical to the initial study with the exception that while in casts for 5 weeks the normal subjects lay on oscillating beds instead of on fixed beds. Over a 2 minute period these beds rock slowly from horizontal to 20 foot down and back to horizontal This bed should not be confused with the rapid rocking bed" which is used for poliomyelitis patients with respiratory involvement. Three of the original four subjects participated The chief finding of interest was the fact that the increases in urinary calcium which occurred during recumbency (Fig 4-4) and the total calcium losses were approximately one-half as great as in the preceding study Repetition of immobilization in a fixed bed in one of the subjects again produced a rather rapid rise in urinary calcium supporting the conclusion that earlier modification of urinary calcium rise had been due to oscillation. An auxiliary finding was that following oscillating bed immobilization recovery of most metabolic and physi

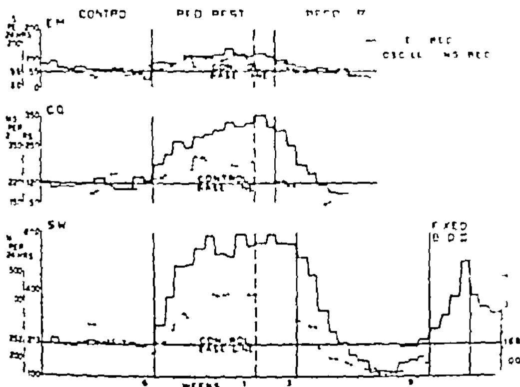


FIG. 4-4 Comparison of the changes in the urinary excretion of calcium resulting from immobilization in fixed and oscillating beds in three normal men. The ordinate scales for urinary calcium excretion (in milligrams per 24 hours) have been placed so that the control base lines of the fixed and oscillating bed experiments are superimposed. The control base line urinary calcium excretion for the fixed-bed experiment was an average of the outputs of the last four control weeks, and for the oscillating-bed experiment an average of the outputs of the last three control weeks; for the reimmobilization of subject in a fixed bed (Fixed Bed II) the control base line was an average of the last two periods (11 days) of the oscillating-bed experiment recovery phase. (*Reproduced by permission of American Journal of Medicine.*)

ologic functions was much more rapid than following fixed bed immobilization.

Study was then undertaken by Whedon and Short of an important seriously immobilizing disease, acute anterior poliomyelitis.[79] These investigations were carried out over a considerably longer period of immobilization and so it was felt would present in a number of respects a more comprehensive picture of changes occurring in bone and calcium metabolism from disuse than did the earlier studies in normal subjects. Altogether 11 patients were observed, 9 of these on a metabolism ward and on constant dietary intakes for periods ranging from 5 to 9 months. Figure 4-5 indicates for each of these 9 patients the duration of study and the phase of disease during which study was carried out. In 4 of the 9 investigation was begun within the first week of the disease. Muscle paresis was extensive in all patients, 7 of the 9 having all four limbs involved. Time of

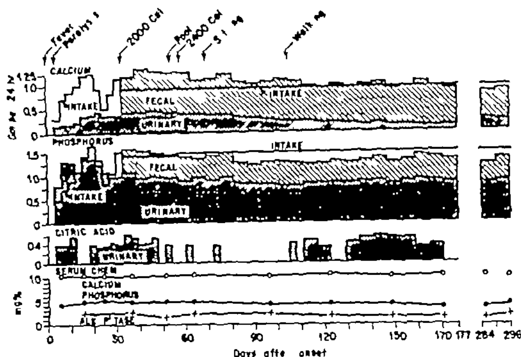


FIG 4-6 Calcium and phosphorus balances, urinary citric acid and serum calcium, phosphorus, and alkaline phosphatase during the first 6 and 10 months of illness in a 21 year-old man with paralytic acute anterior poliomyelitis. Serum alkaline phosphatase is plotted in Bodansky units. (*Reproduced by permission of the Journal of Clinical Investigation*)

the mean serum calcium values for the whole group however correlation was found to be highly significant between serum levels and each of the following total calcium loss, maximal negative calcium balance and maximal urinary calcium excretion Serum alkaline phosphatase generally remained within normal limits, yet tended to be low in the two patients with the smallest calcium losses, and some slightly elevated values were noted in the two patients with the greatest losses Ambulation did not cause an increase in alkaline phosphatase

In view of the widely varying estimates of the percentage loss of total body calcium necessary for osteoporosis to become roentgenographically evident and the lack of specific data on this point, analysis in this respect was made of the total calcium losses of these patients. In the four patients whose studies were begun within 1 week of onset the entire loss was exactly measured in two others all but the small losses of the first three weeks were known X rays of long bones and spine were taken monthly Initial x-ray evidence of osteoporosis was usually a patchy demineralization and coarsening of trabecular markings in the lower extremities regional to the joints, most often in the necks or distal ends of the femora In three patients the first abnormality noted was a submetaphyseal band of rarefaction in the distal tibiae (Fig 4-8) As immobilization continued, areas first noted

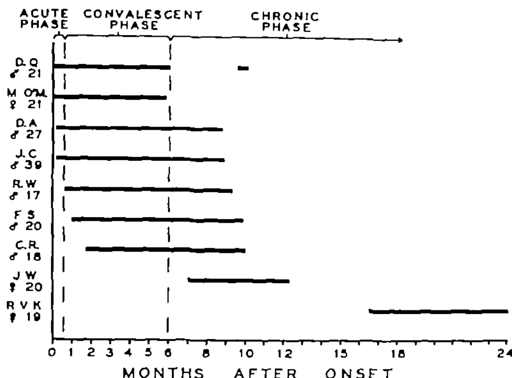


FIG 4 5 The phases of acute anterior poliomyelitis during which metabolic studies were carried out in nine patients, and the duration of these studies in each case (*Reproduced by permission of the Journal of Clinical Investigation*)

mobilization varied rather widely, but the earliest mobilization to a chair was after 2½ months and to walking 3½ months the average period re quired before walking could be begun was 6 months for 7 of the patients the other 2 progressed only to a wheelchair during the course of the study

The principal findings with respect to calcium metabolism were as follows Progressive increase in calcium excretion was noted, primarily in the urinary component, which displayed a pattern similar to that seen pre viously in immobilization of normal subjects at the fifth week after onset of illness urinary calcium reached high levels which were sustained in plateau fashion for many weeks Graphs of the data of two patients (Figs. 4-6 and 4-7) picture the elevation in urinary calcium and the long duration of negative calcium balance the calcium intake in all these patients wa 920 mg/day Specifically (Table 4-1) among the 7 patients studied during the acute and convalescent phases of the disease the mean maximal urinary calcium was 574 mg/day and urinary calcium on the average remained greater than 250 mg/day for 5 5 months. The mean maximal negative calcium balance was greater than half a gram per day calcium balance remained negative on the average for 7 months

With respect to changes in blood chemistry hypercalcemia was noted in 4 of the 7 patients without correlation with the age of the patient. Taking

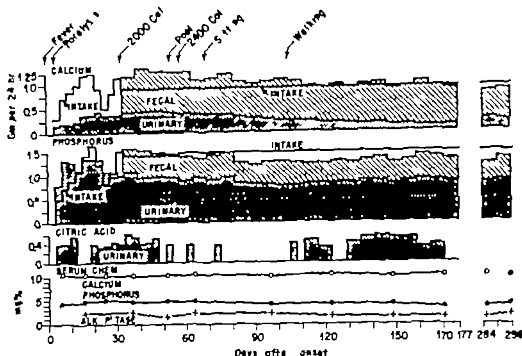


FIG. 4-6. Calcium and phosphorus balances, urinary citric acid and serum calcium, phosphorus and alkaline phosphatase during the first 6 and 10 months of illness in a 21 year-old man with paralytic acute anterior poliomyelitis. Serum alkaline phosphatase is plotted in Bodansky units. (*Reproduced by permission of the Journal of Clinical Investigation*)

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Table 4-1

SUMMARY OF DATA ON CALCIUM EXCRETION AND BALANCE DURING CONVALESCENT PHASE OF PARALYTIC ACUTE ANTERIOR POLIOMYELITIS

Patient	*Maximal urinary calcium (6-day period)* Gm/day	*Week after onset*	*Mean urinary calcium 2d-3d months, Gm/day*	*Duration urinary Ca over 0.250 Gm, months*	*Maximal negative balance* Gm/day	*Week after onset*	*Mean negative balance 2d-3d months, Gm/day*
D Q	0 460	7th	0 401	3.5	−0 420	6th	−0.277
M O M	0.391	5th	0.354	3 5	−0 471	10th	−0.347
D A	0 470	5th	(0.307)	3	−0 420	6th	(−0 124)
J C	0.504	3rd	0.515	5	−0.536	11th	−0 434
	(0.530)	(6th)					
R. W	0 601	5th	(0 477)	6	−0.572	6th	(−0 428)
F S.	0 0 4	6th	0.612	8	(−0 603)	(7th)	−0.560
					−0 609	9th	
C R	0 780	(9th)‡	0 087*	0.5	(−0.605)	(9th)	−0 575
					−0 683	13th	
Mean	0.5 4	5th	0.514†	5 5	−0.540	9th	−0 430†

* Procedure given during second month which reduced calcium excretion.
† Excluding the data of D A. and R. W
‡ Study not started until eighth week.

became more lucent and changes appeared in pubic and ischial rami and in the acetabular fossa of the innominate bone (Fig 4-9) Table 4-2 shows *the quantitative relationships between calcium loss and demonstrable osteoporosis The pertinent figures indicate that the earliest signs of de*mineralization appeared approximately 3 months after onset and occurred with an average loss of 2 0 per cent of the total body calcium No changes were detected in the x rays of the spine although recumbency continued for an average of 6 months negative calcium balance for 7 months, and total calcium losses averaged 4 6 per cent of total body calcium, with a range up to 9 4 per cent

With respect to the 2 per cent figure for proportion of calcium loss for osteoporosis to be first detected, it has been suggested that a higher figure would result if nonparalyzed areas were excluded from the estimation of total body calcium *on the assumption that calcium loss and osteoporosis* would develop only in paralyzed areas Theoretically this would give a better estimate of the percentile calcium loss from any given area for osteoporosis to be detectable by x ray However the average 2 per cent figure can be raised relatively little by this means since in this group of patients

Table 4 2

QUANTITATIVE RELATIONSHIP BETWEEN CALCIUM LOSS AND DEMONSTRABLE OSTEOPOROSIS IN PARALYTIC ACUTE ANTERIOR POLIOMYELITIS

Patient	*Pre illness body weight kg*	*Est total body Ca* Gm*	*Initial long bone osteoporosis*			*Total months recumbent*	*Total calcium loss*		
			Months after onset	*Ca loss Gm*	*Ca loss %*		*Duration months*	*Amount Gm*	*Per cent*
D Q	61	1,008	2	18.0	1 0	3 5	5	27.0	2.5
M O M	3	1 020	3	27 1	2.6	3.5	5.5	47.0	4.3
D L	59	1 007	3.3	11 4	1.0	6	[illegible]	17 0	1 1
J C	82	1 4 0	2.5	27 6	1 0	7	7	54.9	3 [illegible]
R. W	68	1,224	3.3	36.0	2.9	5	8	53.3	4.5
F S.	82	1 4 0	3	33.1	2.2	8	8.5	0.5	0.5
Mean of 6 patients			3	20.2	2 0	5.5	[illegible]	48.6	3.8
C R.	67	1,206	2	(18.0)†	(1.5)†	9	10	113.4	9 4
Mean of 7 patients						6	7	58.0	4.6

* See text and reference 70 for estimation as per cent of body weight.

† Including an estimate of calcium loss of first six weeks of illness based on rate of loss thereafter and taking into account the usual smaller losses during the first three weeks.

only the skull, ribs, and in two instances the upper extremities could be considered unparalyzed.

✓ As I have indicated, no evidence of demineralization in the spine appeared although total calcium losses ranged as high as 9 4 per cent. That early stages of demineralization were present in this area is suggested by observation of other patients with poliomyelitic paralysis in whom demineralization of the spine was detected after approximately one year of immobilization Since these other patients were not on metabolic study however no accurate estimate can be made of the percentage loss of calcium necessary for obvious changes in this area. By comparison with losses of the metabolic group however it may be safely estimated that demonstration of osteoporosis of the spine requires calcium losses of more than 10 per cent.

Now that we have recounted the pattern and degree of calcium loss occurring in this study of paralytic poliomyelitis it seems important to question the extent to which these changes may be safely assigned solely to immobilization or disuse The two other principal influences which might have contributed to the calcium loss are stress, as presumably mediated through the adrenal cortex, and denervation.

The possible complicating influence on mineral loss of hormonal factors related to the adrenal cortex seems relatively easy to treat, at least with

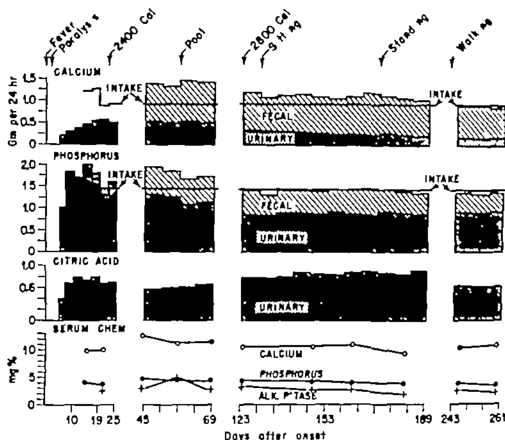


FIG. 4-7 Calcium and phosphorus balances, urinary citric acid excretion, and serum calcium, phosphorus, and alkaline phosphatase during the first, second, fifth sixth, and ninth months of illness in a 39 year-old man with paralytic acute anterior poliomyelitis. Serum alkaline phosphatase is plotted in Bodansky units (*Reproduced by permission of the Journal of Clinical Investigation*)

respect to poliomyelitis. Hormonal influence can be minimized principally on the basis that data thus far obtained (plasma 17-hydroxycorticoids and urinary 17 ketosteroids) suggest that the "stress" influence in poliomyelitis is both modest and brief[19-22] Urinary corticoid excretion measurements if reported in poliomyelitis have escaped our attention Accepting as fact that stress is not an important element in paralytic poliomyelitis would enable us to avoid the question of the influence of adrenal cortical steroids on bone For completeness however the statement should be inserted that the effects of these steroids seem varied and that the opinion that these steroids have a clear-cut osteolytic or calcium-losing effect in human beings has not yet been supported by conclusive metabolic data. Thus far support has been provided only either by rather brief balance observations or by analyses limited to urine[23, 24] the balance studies of the Mayo group on 30-day administrations of cortisone[25] and prednisone[26] to patients with rheumatoid arthritis did not show changes in over all calcium balance

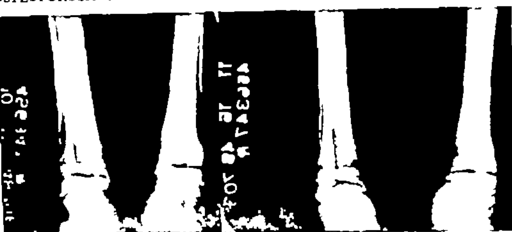


Fig 4-8 Initial x ray evidence of development of osteoporosis in paralytic poliomyelitis. *Left* X-ray of tibiae taken during first month after onset of illness showing essentially normal bone density *Right* X ray of tibiae taken 2 months after onset, showing submetaphyseal band of rarefaction. (*Reproduced by permission of the Journal of Clinical Investigation.*)

even though urinary calcium was increased in some patients Our own balance studies of the metabolic effects of prednisolone are in agreement.[27]

The conclusion that disuse or immobilization unrelated to neural factors is chiefly responsible for the mineral losses is based mainly on the similarity of pattern and degree of these losses in various situations in which immobilization was an outstanding feature such as confinement of normal individuals to bed in casts,[8] leg fractures,[9] and paralytic poliomyelitis [20] The degree of calcium loss, however was greater in the latter two situations (and probably the degree of immobilization was also greater) than in



Fig. 4-9 Later x ray evidences of development of osteoporosis in paralytic poliomyelitis. *Left* X ray of pelvis and femur taken during first month after onset of illness, showing essentially normal bone density *Right* X-ray of pelvis and femur taken 6 months after onset, showing rarefaction of public ramus acetabular fossa and neck and intertrochanteric area of femur (*Reproduced by permission of the Journal of Clinical Investigation*)

the confinement of normal subjects. Of possible further significance to assignment of a major role to disuse was the observation that the magnitude and particularly duration of calcium losses in the poliomyelitis patients were proportional to the degree and duration of immobilization the point is muddied however by the fact that severity of paralysis in general accounted for the degree and duration of immobilization.

To sort out the influence of disuse per se from that due to denervation per se is very difficult, but instances may be cited in which this seems at least partly possible Some of these instances support the predominant influence of disuse or immobilization per se, and some tend to emphasize neural factors First, in our poliomyelitis study four patients had severe paralysis of the upper extremities yet only one of the four showed demineralization of arm bones and that only to a mild extent. On the other hand demineralization was uniformly present in femora and tibiae even though lower extremity paralysis was in several instances no more marked, if not less, than upper Perhaps upper extremities, though severely paralyzed, rarely became demineralized because they were sufficiently used or moved

Further supporting the idea that disuse per se is of primary importance in the causation of calcium loss is the observation of Abramson[28] of the effect of weight-bearing on two groups of paraplegics. Over a 3-year period one group moved about only by wheelchair while the other walked with braces and crutches for an average of 1 hour daily Twenty-five of the thirty nonambulatory paraplegics showed osteoporosis of the pelvis and legs, whereas *only one of the eight who walked with similar injury to the* spinal cord showed bone rarefaction and that to only a mild degree The experimental studies of Gillespie[29] on the effects of nerve root section in *kittens indicated that the reduction in bone weight correlated directly* (+0 79) with the reduction in muscle weight in the affected limbs this led to the conclusion that the bone changes were due to the loss of muscular *activity and that there was no evidence for a specific or trophic influence* of nerves on bone

On the other side of the discussion, however may be cited some *disconcerting observations of the relationships between increased activity or* ambulation and changes in calcium loss in our poliomyelitis study[20] Essentially these are that urinary calcium levels began to decrease uniformly prior to the beginning of standing or walking and that, with the beginning of ambulation, calcium excretion continued to decline gradually and did not show particular evidence of an increased rate of fall In possible explanation of this latter statement, it should be pointed out that in this study ambulation could not be instituted in any abrupt or rapid fashion because of the considerable degree of muscle impairment in these patients. In the study of normal subjects[8] where the change from bed rest to ambulation was quite sharp a definite although sluggish shift from loss toward storage

was noted. An additional possibly negative point with regard to stress and strain may be cited, that is, slow rocking or oscillation was without avail in reducing the calcium loss of the polio patients [29] It may also be mentioned that Wyse and Pattee[30] in a study of paraplegics tried both oscilla tion and weight bearing on a tilt table without affecting calcium balance

A more forceful case for the importance of neural or neurohumoral factors in demineralization associated with poliomyelitis has been made by Dunning and Plum [31] who have noted hypercalcinuria in patients with bul bar involvement and without spinal involvement, who thus could remain ambulatory to variable extents. In addition they felt that the degree of hypercalcinuria noted in more extensively paralyzed patients was unrelated to the amount of muscle paralysis and resultant immobilization although the duration of hypercalcinuria was furthermore, in a later study [32] therapeutic mobilization failed to induce any immediate amelioration of hypercalcinuria and failed to shorten its duration.

The reasons for the conflicting results bearing on this question are not at all clear· the opposite conclusions must reflect, at least in part, either differing techniques or overzealous interpretation of the data obtained It seems evident that more information will be necessary to determine for certain whether there are neural or neurohumoral influences on bone and whether they exert effects which are direct or are merely mediated through the disuse with which they are associated

This discussion of neural factors versus disuse points up the fact that we do not know the exact manner in which mechanical stresses and strains exert their effect on bone mass whether through the medium of muscle pull on periosteal surfaces, or more directly in weight bearing through bone structures and columns, or by combinations of these two and perhaps other factors. The failure of oscillation to influence calcium balance in poliomyelitis or in paraplegia, where muscle function is greatly or totally reduced, in contrast to its effectiveness in immobilized normal subjects may be used to suggest the greater importance of muscle pull The Abram son study of paraplegics, however if valid can be cited in favor of direct bony weight-bearing

The possible effects of changes in circulation to bone in association with disuse also need to be considered. Bone circulation is certainly difficult to measure even if one accepts the idea that adequate techniques for its measurement have been devised That circulatory influences on bone may be important is indicated from various observations in other states Hy peremic circulatory changes have been reported to improve bone growth for example following sympathectomy[33] and in long-standing arteriovenous fistulas,[34] and to accelerate fracture healing, as in experimental venous ligation [35] A conflicting view[36] has been expressed however that decreased blood supply results in increased density of bone and that hyperemia leads to skeletal demineralization It is not known whether changes in bone

circulation occur either during the paralytic stages of poliomyelitis or in uncomplicated bed rest and if so what kind

Review of these various mechanisms through which disuse may exert its effects on bone about which we have so little specific and real data, must lead us to admit that we cannot even be absolutely certain that the basic process is diminished osteoblastic activity It has not been really ruled out that during immobilization the increase in calcium excretion and hypercalcemia may actually reflect an increase in bone resorption. It is to be hoped that this heretical question will be resolved clearly by the radioactive techniques which Bauer[37] ourselves,[39] and others have been using to determine rates of bone formation

As a final section of these remarks brief mention may be made of considerations which are predominantly clinical and therapeutic. In patients with paralytic poliomyelitis, the degree of mineral loss can be definitely reduced by administration of testosterone propionate alone or in combination with estradiol benzoate[30] (Fig 4-10) Our studies indicated no clear advantage from combined steroid administration over testosterone alone

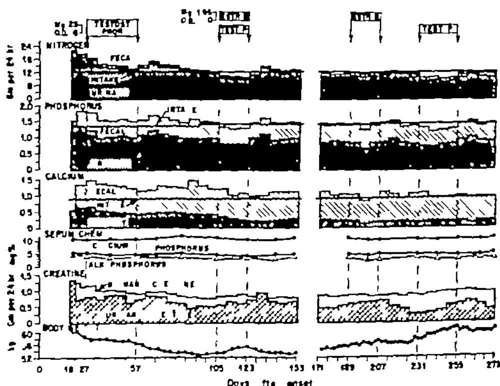


FIG. 4-10 Metabolic data on a patient with paralytic poliomyelitis during the first nine months after onset of paralytic acute anterior poliomyelitis, showing the effects of testosterone propionate given during the early convalescent and chronic phases and of combined steroid and estradiol benzoate given during the convalescent phase (*Reproduced by permission of the Journal of Clinical Investigation.*)

but the addition of estrogen has usefulness in tending to prevent urinary calculus formation by increasing urinary citric acid and counteracting the lowering effect of testosterone on this constituent Data on oral gonadal steroids are scant, but Plum[19] has noted the usefulness of 17-ethyl 19 nor testosterone in lessening calcium excretion in poliomyelitis patients as we and others have in senile osteoporosis. Sodium phytate has been suggested[40] as a means of reducing urinary calcium excretion by diverting the calcium to the feces in modest doses phytate does not appreciably alter calcium balance On the other hand, little influence has been noted of various levels of calcium dietary intake on urinary calcium in paraplegic patients[41] this is of interest in view of the general admonition to keep calcium intake low for fear of calculus formation an instruction which accentuates the negativity of calcium balance Concern for the effect of intake on urinary calcium levels probably can be relaxed somewhat, particularly if hormonal or other agents are used to retard urinary calcium excretion

Summary and Conclusions

In uncomplicated studies in normal subjects of the effects of disuse or immobilization, calcium losses have been observed which, if continued, would be expected to lead to the acute development of osteoporosis In studies of paralytic poliomyelitis apparently involving more marked immobilization, greater calcium losses were noted as well as the development of osteoporosis which was quantitatively related to calcium loss Assignment of these greater calcium losses mainly to disuse has raised questions of the presence and importance of direct neural influences on bone The resulting dissension seems to have unmasked the shallowness of our knowledge of various facets of the subject, disuse atrophy of bone We may take partial comfort, however from recent therapeutic suggestions which should prove useful in the management of conditions leading to or involving this type of osteoporosis

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DISCUSSION

Osteoporosis

Comments by the Chairman Edward C Reifenstein Jr M.D

There has been considerable discussion about some of the concepts with which I have been concerned over the years Therefore I shall take about 5 minutes to present one or two points about my theories particularly those which Dr Urist mentioned [1 2]

In 1940 Dr Fuller Albright (with whom I was associated) was concerned particularly with the development of anabolic steroid deficiency in aging people and pointed out that osteoporosis in older individuals may be a manifestation of this deficiency At that time, he recognized that there were other factors involved because not all persons who had a deficiency of anabolic steroids exhibited clinical evidence of osteoporosis [4-7]

More recently with the improvement of techniques, it has been possible to make some important measurements which provide some further insight into the problem of osteoporosis. Data from one of these investigations are presented in Fig 4-11 which I have prepared by calculations from the data published by investigators at the Worcester Foundation for Experimental Biology [8-10] These workers have studied by decades the urinary excretion of androgenic compounds (as androsterone in milligrams per hour) the urinary excretion of estrogenic substances (in rat units per 24 hours) and the urinary excretion of neutral reducing lipids (in milligrams per 24 hours) These excretion values are used as indices The excretion of androgen and estrogen together is assumed to be an index, if not a measure, of the production of the anabolic type of steroids by this I mean those steroid compounds which stimulate the anabolism of protein and osseous tissues. The excretion of neutral reducing lipids is assumed to be an index, if not a measure of the production of the glucocorticoid type of adrenal cortical steroids As far as protein is concerned I believe that such corticosteroids are initially antianabolic in action and if given in large amounts and/or for long periods they may become catabolic in action It is the antianabolic action of these compounds that I am considering here

The data as presented in Fig 4-11 give an indication of the total amount of anabolic steroids excreted by women and by men during the decades from 25 to 75 years of age The calculations make it possible to compare the amounts excreted by the two sexes

The graphs were constructed after the following steps (1) the combined excretion of androgenic and estrogenic activities is assumed to be an index, if not a measure of the production of anabolic steroids (2) the urinary estrogenic activity excreted at various ages was calculated for each sex in percentage of the amount excreted by women at the age of 25

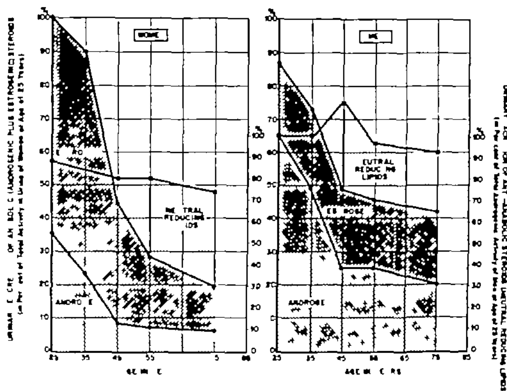


FIG 4-11 The effect of age and sex upon the urinary excretion of steroids with anabolic activity (androgen and estrogen) and with antianabolic activity (neutral reducing lipids) (*Reifenstein Clin Orthop.*, 10 206–253 1957)

years (3) the urinary androgenic activity excreted at various ages was calculated for each sex in percentage of the amount excreted by men at the age of 25 years (4) then for each sex the percentage excretions thus derived were added at the respective age periods (5) the resulting total excretion of androgenic and estrogenic activities was recalculated for each sex in percentage of the total quantity of steroids with these activities excreted by women at the age of 25 years (6) the resulting values were charted (7) the urinary excretion of neutral reducing lipids is assumed to be an index if not a measure of the antianabolic steroid excretion (8) the approximate excretion at various ages was calculated for each sex by averaging the values for the sleeping waking and day collections of the original study (9) the urinary antianabolic activity excreted at various ages was calculated for each sex in percentage of the amount excreted by men at the age of 25 years (10) then for each sex the activity values were recalculated as the percentage equivalent of the androgen excretion of men at the age of 25 years and (11) the resulting values were charted.

The graphs show that, with declining years (1) the total production of anabolic steroids diminishes in both sexes (2) the decreased production occurs at an earlier age is more precipitous and reaches a considerably

lower level in women than in men (3) the production of antianabolic steroids diminishes only slightly in both sexes and (4) the more marked decrease in the production of anabolic steroids results in a relative excess of antianabolic steroids over anabolic steroids which is more rapidly induced and considerably greater in women than in men Thus the aging individual develops a steroid imbalance qualitatively similar to that of Cushing's syndrome.

These data therefore support the concept of 1940 that there is a decrease in the production of anabolic steroids with age in women and also in men that the amount of decrease is more marked in women than in men, and that these relationships are correlated with the earlier onset and the more severe nature of the osteoporosis seen in women[4, 7] At that time we were not paying particular attention to what was happening to the adrenal corticosteroid hormone production However the recent measurements indicate that the adrenal corticosteroid production (as shown by the index of the neutral reducing lipid excretion and by other studies) undergoes only a very small reduction with aging In other words, in the women it falls only about 10 per cent and in the men it falls barely 5 per cent from the initial level

In the 1956 concept, I have been emphasizing the fact that there always is a relative excess of the corticoid (antianabolic) to the anabolic steroids in the older person[1-3] We had given little attention to this point in the past. However I now believe that this alteration forms a background which may be very important for the development of osteoporosis This point is illustrated in Fig 4-12 It will be seen that there is a balance between the hormonal influences in the normal adult (diagram *A*) that there is an absolute increase in the antianabolic action in Cushing's syndrome and in the normal adult receiving chronic corticoid therapy (diagrams *B* and *C*) and that there is a relative increase in the antianabolic action (as a result of a decrease in the anabolic action) in old age and in the postmenopausal woman (diagrams *D* and *E*)

At some point in life for example in the normal adult of 25 years of age the opposing influences on protein tissues are balanced with the anabolic and the antianabolic steroids in equilibrium In Cushing's syndrome, we have an endogenous excess of corticosteroids which creates an imbalance consisting of more antianabolic than anabolic steroids Osteoporosis occurs very commonly in Cushing's syndrome When we give an adult whose steroids with these opposing activities have been in balance an excess of corticoids as therapy we create the same type of imbalance as in Cushing's syndrome. After corticoid therapy for considerable periods of time and/or in large doses, such individuals develop osteoporosis and spontaneous fractures In old age, we have a decrease in the production of anabolic steroids This is the aspect upon which we were focusing in 1940[4, 7] In 1956, I began to direct more attention to the fact that simul-

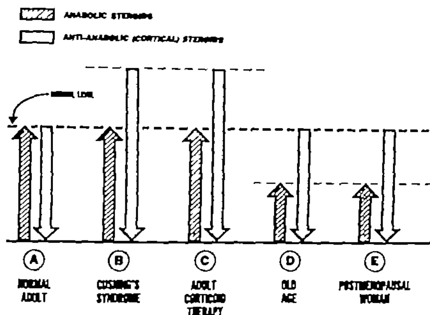


Fig 4-12. Schematic diagrams of the balance between anabolic and antianabolic steroids in various physiologic and pathologic states. An arrow pointing upward represents a stimulating effect, an arrow pointing downward represents an inhibiting effect· the length of an arrow indicates the amount of effect. The hatched arrows indicate anabolic steroids the stippled arrows, cortical steroids. The heavy horizontal dash line is the normal level The relation between the endogenous levels of anabolic and antianabolic steroids is shown for five states *A* the normal adult, *B* Cushing's syndrome *C* the normal adult receiving chronic corticoid therapy *D* old age· and *E* the postmenopausal woman (*Reifenstein South M J* 49 933–960 1949)

taneously in old age there is a relative excess of antianabolic adrenal cortical steroids which results in an imbalance qualitatively similar to that in Cushing's syndrome and which therefore might set the stage for the development of osteoporosis.[1 2]

Next, I wish to indicate clearly the point upon which Dr Urist and I seem to be differing slightly It is my concept that the imbalance between the anabolic and the antianabolic steroids occurs in all individuals as they age and that there is still a *factor* which we have not identified, in bone which makes the osseous system of some individuals more susceptible than that of others This may be the "antiosteoporosis" *factor* to which Dr Urist refers it may be the low calcium intake that Dr Nordin has been speaking about it could be the amount of activity which Dr Whedon discussed it could be the amount of protein intake over a long period of time it could be a combination of these factors or it could be some other currently obscure and unrecognized influence alone or in conjunction with these various factors In contrast Dr Urist believes that between the persons who have clinical senile osteoporosis and those in the same age group who do not he will find a qualitative or a quantitative difference in

the hormone imbalance itself (and particularly in the adrenal cortical steroid components)

The data which have been accumulated thus far support the contention that the imbalance occurs in all aging persons. Dr Urist s own studies con firm this observation because in all the measurements which he has carried out thus far he has found no difference between those who are osteoporotic and those who are not Dr Urist has been looking for an alteration in the hormone balance which would be a specific determining influence for the development of osteoporosis I consider the hormone imbalance as a general nonspecific predisposing influence which sets the stage for the development of osteoporosis. It is my guess that the difference in those who get clinical osteoporosis as they age is in the bone itself rather than in the endocrine glands or their hormones

Next, I would like to make clear our concept of the pathologic physi ology of what we call osteoporosis since our concept (which is the one that Dr Albright proposed) is not entirely the same as that of Dr Nordin. I believe that to a large extent this difference in the concepts accounts for the difference in the results Dr Nordin has presented and in those we previ ously reported Our concept is summarized schematically in Fig 4-13

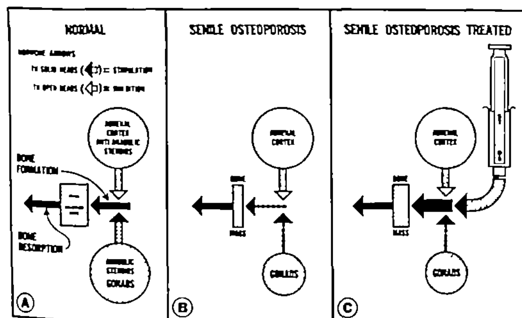


FIG. 4-13 Schematic diagrams illustrating the relationships of steroid hormones to the development and the treatment of senile osteoporosis. (*Reproduced by permission of Reifenstein Clin Orthop* 10 206–253 1957)

In this chart the rectangle is the mass of calcified bone the black arrows are the processes of bone formation and bone resorption with the size of the arrows representing the rates of these processes the circles are the adrenal cortex as a source of antianabolic hormones and the gonads

as a source of anabolic steroids the stippled arrows are the antianabolic steroids and the cross hatched arrows the anabolic steroids with the size of the arrows representing the amount of hormonal influence the arrows with open heads are hormones with an inhibiting action, and those with solid heads are hormones with a stimulating action the syringe labeled "anabolic steroids" represents administered anabolic therapy The chart has three divisions (*A*) the normal status, in which the calcified mass of bone is in a state of dynamic equilibrium (because the rate of bone formation is equal to the rate of bone resorption) and the endogenous hormonal influences upon bone formation are in balance, (*B*) senile osteoporosis, in which the mass of calcified bone has decreased (because bone formation is reduced) and the endogenous hormonal influences upon bone formation are altered so that there is an absolute deficiency of anabolic steroids and a relative excess of antianabolic steroids and (*C*) senile osteoporosis treated with anabolic steroid therapy in which the calcified mass of bone is being increased toward normal by an increase in bone formation and the imbalance between the endogenous antianabolic and the anabolic hormones is being corrected by the administration of anabolic steroid therapy

In the normal individual there are two processes, bone formation and bone resorption, going on simultaneously at various sites in the skeleton at such rates that the amount of mineral which goes into the calcified bone mass is equal to the amount of mineral which goes out of the mass therefore, *the calcified bone mass is in a state of dynamic equilibrium.* As we see it, the rate of the process of bone formation is under the influence of certain hormonal factors those with anabolic actions and those with *antianabolic factors The anabolic steroids tend to increase* the bone forming activity In Fig 4-13 I have shown the gonads as one source of anabolic steroids, but I recognize that the adrenal cortex also is a source of similar anabolic steroids. At the same time, the effect of the anabolic steroids in increasing the rate of bone formation is held in check by an opposing influence, the inhibiting or antianabolic effect of the adrenal glucocorticosteroids on the rate of bone formation so that a balance exists between the hormonal influences which permits a normal rate of bone formation to be maintained

We all agree that in senile osteoporosis there is a decrease in the total amount of calcified bone mass In this condition, according to our concept, there is primarily a defect in the rate of formation of calcified bone because of a defect in the rate of formation of the protein osseous matrix (which must be deposited before bone tissue can be calcified) In our concept, a decrease in the rate of bone formation which leads to osteoporosis can be related in part to a decrease in the amount of anabolic steroids, to a relative excess in the amount of *antianabolic steroids or most probably* to the occurrence of both alterations. Dr Nordin defines osteoporosis as a

corticoids of only about 10 per cent, whereas there is a marked decrease in the amount of anabolic steroids of about 50 to 80 per cent (50 per cent in males and 80 per cent in females) Thus there is an imbalance be tween the anabolic and the antianabolic steroids This imbalance could be corrected by therapy in several ways

1 We could overcome the deficiency in the endogenous anabolic steroid level by administering anabolic steroid compounds This is what we have been doing since 1940 This type of therapy restores the anabolic anti anabolic hormone balance and arrests the progress of the osteoporosis from a practical point of view it does not "cure" the condition The dif ficulty which all of us have experienced, is to bring about the development of a recognizable increase in the mass of calcified bone This difficulty may result from our inability to create a sufficient excess of anabolic over anti anabolic influences. In a few cases which seem to be well documented a recognizable increase has been reported this could result from the fact that, when the anabolic level is restored to that which is consistent with a maximal tissue response (i e with the full response of a person of 25 years of age) the antianabolic level has decreased with age by 5 to 10 per cent, and thus there is an anabolic excess of 5 to 10 per cent. In any event, this advantage in favor of the anabolic influences apparently is not suf ficient for practical curative" therapy since it usually takes at least 10 to 15 years to achieve a recognizable increment in the calcified bone mass

2. We *could reduce the relative excess of endogenous antianabolic* steroids by administering an ACTH inhibiting agent which, in itself is not a corticoid. At present, we do not have a compound with this activity which is safe to administer However this is a therapeutic agent to be sought. This type of therapy would restore the anabolic-antianabolic hor mone balance and would arrest the disease again from a practical point of view it would not "cure" the condition

3 We could reduce the relative excess of endogenous antianabolic steroids as in the previous method of treatment and then administer in addition anabolic steroids in sufficient amounts to bring about a maximal tissue response This would create a considerable excess of anabolic over antianabolic influences Such an excess might permit the development of new units of calcified bone mass at a rate which would bring about an increase in the total amount of calcified bone sufficient to result in a "practi cal cure" of the senile osteoporosis in a reasonable period of time

* * *

CHAIRMAN REIFENSTEIN We will now proceed with the question and answer period. The following question has been directed to Dr Whedon "Were normal individuals subjected to immobilization? We are not clear as to the type of individuals who were subjected to immobilization in your study "

DR. WHEDON Yes, they were four normal healthy subjects, aged 20 to 29 in the first study I described After the immobilization of normal healthy young men, in the second phase of my presentation I showed the metabolic changes in patients with paralytic poliomyelitis

CHAIRMAN REIFENSTEIN Here is a question from V C Turner for Dr Sissons "Have any studies shown that long-continued use of corticosteroids in children affects growth?"

DR. SISSONS Yes I would draw your attention to the work of Blodgett and collaborators (see Chap 1) These workers made regular measurements of height in children receiving therapeutic doses of cortisone for hormonal abnormalities or allergic disorders. They found that as little as 4 to 20 mg per square meter of body surface per day had a marked growth suppressing effect in hypopituitary patients, that about 35 mg/sq m/day was needed to reduce the rate of growth of patients with adrenocortical virilism to average normal levels while a minimum of 45 mg/sq m/day was required to reduce the growth of endocrine normal children

DR NORDIN I have been asked two questions here "Were the dietitians making the estimates of calcium intakes ignorant of the classification (normal or osteoporosis) of subjects?"

No I am afraid in many cases they were not, for the simple reason that many of the patients were referred from my own clinic which happens to be on a Thursday and if the patients came to the dietitian on Thursday afternoon, an intelligent dietitian might reasonably draw the conclusion that I suspected the patient of having osteoporosis On the other hand many of the patients that had been sent to me with backache did not have radiologic osteoporosis and so were not in the series They were included only if they had x ray evidence of osteoporosis, but the dietitians did not see the x rays.

Although I think there is a possibility of bias here, I hope it has not been a large one and I have spoken to all the dietitians individually and pleaded with them not on any account to try to help my work by producing figures which they think are going to support my arguments I think my colleagues at the Western Infirmary have also made this same point to the dietitians in no uncertain terms, and I think the result is that the study is reasonably unbiased

I have also been asked to comment on the high accretion rates in the normal subjects They are I agree a bit higher than were reported in normal subjects by other people I don't know the explanation for this, but perhaps when I have had a chance to talk with Dr Bauer this evening I will be able to let you have the answer

CHAIRMAN REIFENSTEIN Dr Urist, one of the audience has asked you to restate your message Another wanted to have your conclusion given again

DR. URIST The conclusion of my paper is that the cause of osteoporosis is not known and that there is an endogenous factor other than estrogen,

androgen and glucocorticoids in the blood that is responsible for osteoporosis. It is this unknown factor that I for purposes of a working hypothesis call the antiosteoporosis factor This is what distinguishes the patient of comparable age without osteoporosis from one with it. The Reifenstein Albright hypothesis of 1956 the anabolic-antianabolic hormone imbalance hypothesis, is unproved however if the case were presented in a court of law the bulk of evidence, I think, would be in favor of the endocrine rather than the dietary deficiency factors as the cause of osteoporosis

The greatest need at present is an adult laboratory animal with experimental osteoporosis The Canadian goose is an animal in which osteoporosis occurs spontaneously at the conclusion of a period of reproduction. In this species, it appears that we have an experimental animal with spontaneous osteoporosis. Osteoporosis, as it appears in man, has not been *produced in the rat. Attempts to produce it by gonadectomy have failed* apparently because the adrenal takes over the function of the gonads and produces gonadal hormones (the adrenal will increase in size two or three times in gonadectomized animals) This experiment is being done in our laboratory with the adult rat, receiving a small maintenance dose of cortisone, following adrenalectomy and compared with gonadectomy or adrenogonadectomy I do not have time to go into experimental osteoporosis, but it seems necessary to emphasize the point that more work on this subject should be done in the laboratory as well as in the clinic.

CHAIRMAN REIFENSTEIN Dr Whedon, do you want to answer the questions which have been directed to you?

DR WHEDON The first one is "Do changes in renal blood flow influence urinary calcium excretion and is renal blood flow affected by the supine position?"

The answer is that there are definite renal blood flow changes with changes in posture and renal blood flow is increased in the supine position in comparison with the motionless upright position This observation, however is not really pertinent to the matter at hand what is important is whether renal blood flow is altered between the ambulatory upright position and the supine This question has not been answered directly probably but the inference of studies along this line is that there is no appreciable or impressive difference in the renal blood flow between these two positions The effect of changes in renal blood flow on urinary calcium excretion has not been studied to my knowledge

Here is another question "In your body-cast normal patients, were measurements made of the effects on respiration (tidal volume pH CO_2 and so on)?"

Measurements of respiratory function to the extent of tidal volume, vital capacity maximum ventilation capacity and breath holding were made and no changes were noted between the control state and the im

mobilized state nor were there any in the oscillated normal immobilized subjects.

Measurements of blood pH and CO_2 were not made I think the questioner has in mind hypercapnia from diminished ventilation as in paralytic poliomyelitis and that some very definite increases in ventilation can be brought about by rapid rocking All I can say on this is that the oscillation that was presented to you today was the very slow oscillation of the Sanders bed, used in peripheral vascular disease This was not a study of the ventilatory supportive type of rocking bed that is used in the paralytic poliomyelitis patient with impaired muscles of respiration and ventilation.

"Were any of the poliomyelitis patients chronically hyperventilated to see the effect on calcium?" is the third question

They were not I really don't know of studies that would cover that question, nor do I see its point in the present discussion

Finally "Why does immobilization cause more bone atrophy in some patients than in others with about equal immobilization and so on?"

I think this hits at the heart of what Dr Reifenstein was just discussing We don't yet know but there must be some very definite differences from one individual to another in the way they handle calcium These differences may be in part on a hormonal basis, in part dietary and the dietary history over the years may indeed, influence the manner in which a patient responds, or appears to respond by the crude measurement of x ray to immobilization Our interest, similar to that of Dr Nordin in the influence of dietary calcium seems to be leading to results which indicate wide differences in the intake level at which various individuals are in calcium equilibrium or balance osteoporotics requiring higher intake levels. The *mechanisms underlying these differences we are in the process of trying* to find out.

CHAIRMAN REIFENSTEIN Dr Sissons, would you like to carry on with the next question?

DR SISSONS Dr Costich asks "In Storey's work on the experimental administration of cortisone to rabbits did not the exaggerated bony resorption result from forces applied to the teeth with a steel spring?" You will recall my noting that, in the skull and tooth sockets of rabbits receiving cortisone Storey noted the presence of bony rarefaction as indicated by an increased number of resorption cavities, some of which contained osteoclasts, and that he interpreted the results as indicating very rapid resorption of bone In one of Storey's experiments [11] abnormal pressure was indeed applied to the upper front incisor teeth by means of a coiled spring inserted into fine holes drilled through the crowns of the teeth *In control* animals, such mechanical displacement of teeth produces bony resorption on the sides of the tooth sockets subjected to pressure after the administration of cortisone this bony resorption is exaggerated In another of Storey's

experiments on the results of cortisone administration, however[12] the teeth were not subjected to mechanical displacement, and other parts of the skeleton than tooth sockets were studied In this work too Storey's conclusion was that cortisone administration resulted in exaggerated bone destruction The first point I made was that, in order to be quite sure about this, one would need to have more information on the structural changes that might be expected to follow a "normal" amount of bone destruction in the absence of bone formation My second point was that in order to do this we needed information on the fractions of the total bone surface occupied by bone formation and bone destruction. This was a general point that I would like to emphasize this is the type of histologic information which together with an estimate of accretion rate (the g' mentioned in Chap 1) we need in order to determine skeletal turnover and to assess independently the alterations in bone formation and bone destruction How nice it would be to set up an experiment studying the effect of cortisone administration on normal adult bone and, using isotope measurements, make histologic studies and over all metabolic studies in order to have a number of independent estimates of the processes concerned! It would be very exciting to know that they all balanced up and gave us the same answer

DR NORDIN I have been asked a question here which I think goes to the root of the matter so far as I am concerned The question is, "Is not osteoporosis the result of an inability to adjust the metabolism to a low calcium intake? Surely many normal people with less than 14 mg/kg intake are not suffering from osteoporosis"

That is an excellent and reasonable question, and the answer involves the crux of the whole argument. There is plenty of evidence from long-term balance studies that some normal people or the majority of normal people can adjust to a somewhat lowered calcium intake There is the work of Fox, Walker and Irving, there is the work of Nicolaysen and there are a number of other studies which tend to show that if you put ordinary men and women on an intake of 500 mg of calcium a day or 400 mg a day they will adapt, or most of them will adapt The whole point I am trying to make is that osteoporosis occurs in those people who fail to adapt, and if you look at these studies you will find that not all the subjects have adapted Everyone assumes that, because most people can adapt, all people can adapt, and the point I want to make is that those who cannot adapt are the ones who in the long run get osteoporosis

For instance in Nicolaysen's paper there are subjects who were on low calcium intake for over a year who at the end of that year were still in negative calcium balance Those are the kind of people who in my opinion are bound to get osteoporosis The negative calcium balance is bound to take place at the expense of bone There is nowhere else that it can come from and if they are destroying bone they end up with diminished amounts

of bone, and unless you believe that all negative calcium balance produces osteomalacia, which is extremely unlikely the lesion these people must get is osteoporosis

If one could take 1 000 diet histories from subjects instead of 100 or 92, one would find I have little doubt, that there is a distribution for the calcium intake of normal people and if you could do 1 000 urine calcium outputs on normal people you would find that there was a distribution there as well

The point I want to drive home is that unless the people on the low intakes happen to be the ones with low output, and the ones on the high intakes the ones with high outputs, some of these people must inevitably be in negative balance Some of the people who are at the top of the output scale, even if only 1 in 100 or 1 in 1 000 may happen to be (and in fact, I believe my data suggest that they are) some of these people who are at the bottom of the input scale and these people must be in negative balance

In my opinion the reason why this has not become more obvious earlier is that there is a great deal of calcium in the skeleton This process of negative balance will take 20 or 30 years to produce x ray changes in the skeleton, and adaptation to low calcium intake hasn t got a very great survival value

If you go on a low sodium diet, the urine sodium drops within 4 or 5 days. If it didn't, you would get Addison s disease But if you go on a low calcium intake the adaptation at best is slow even in normal people It is slow and it is halfhearted. Any of you put yourself on a low calcium diet, and your urine calcium will drop from 300 to 225 mg or something of that order It won t do as sodium does, it won't go down from 100 mEq to 10 mEq a day It is of quite a different order and quite a different power of efficiency and I think that the people who get osteoporosis are the ones who adapt poorly

CHAIRMAN REIFENSTEIN I would like to ask you one more question Dr Nordin In the patients you call osteoporotics who developed a positive calcium balance on a high calcium intake was the high calcium diet offsetting a mild secondary hyperparathyroidism induced by the chronic low calcium intake?

DR NORDIN This is another very central question I do not believe that there is any such thing as secondary hyperparathyroidism due to low calcium intakes The central feature of my argument is that the blood calcium level the plasma calcium level, is not affected by variations in dietary calcium intake. Therefore because it is not affected, at least to the extent that we can measure not affected appreciably there is no parathyroid stimulation

So far as direct evidence of this is concerned, I may say that some years ago when I thought that low calcium diets would stimulate the parathyroids I went around the wards placing patients on low calcium diets and meas-

uring their phosphate clearances at weekly intervals, and I was increasingly disappointed and frustrated because the plasma phosphate level never fell and the urine phosphate output never rose In other words, secondary hyperparathyroidism did not and does not occur from a low calcium diet.

This is no longer a mystery to me Secondary hyperparathyroidism occurs only if there is a substantial or real lowering of blood calcium level, such as occurs in vitamin D deficiency or with the raised phosphate level of chronic renal insufficiency In those two conditions, there is a real lowering of plasma calcium and, as a result, there is a stimulation of the parathyroid but in the absence of those two conditions ordinary low intake straight malabsorption, or straight hypercalcinuria, none of these three things has, in my opinion, any effect on blood calcium level such as to stimulate the parathyroids.

DR. URIST This is a question that has some bearing on Dr Nordin's thesis "Dr Urist, did your patients show a statistical difference in calcium intake? Were the patients receiving hormonal treatment?"

The most informative group of our 837 patients were the 100 ladies of the Eastern Star Home (the average age was 85 and 26 per cent had severe osteoporosis) There are very good records of the diet of these patients The ladies were classified in three groups those who were taking more than 800 mg a day those preferring between 200 and 800 mg, and those selecting a diet containing under 200 mg a day Of the 26 per cent with severe osteoporosis, 7 per cent had had a higher calcium intake than the daily average adult requirement. Only 8 per cent had a low calcium intake because of an aversion to milk, an alleged allergy or complaints of distention or constipation Approximately 85 per cent had the same calcium intake as the 74 women without osteoporosis Our impression of the factor of diet is that low calcium with normal or high vitamin D intake is bound to aggravate osteoporosis, but it probably does not cause this disorder as we see it in the United States

I have avoided saying anything about treatment because as an orthopedic surgeon, like many others of my specialty I see the patients at the stage when they are disabled by a fracture Some of these patients received steroid hormone treatment and others did not Some received treatment for a short period and declined to continue it because of side effects such as arousal of libido menstrual bleeding hoarseness of voice, acne, hirsutism etc The best information on the subject of treatment has come from endocrinologists and nutrition specialists who pursue a logical and prolonged course of therapy However the majority of patients hospitalized with osteoporosis are on orthopedic wards and are immobilized with fractures of one kind or another In this group the osteoporosis receives secondary consideration and is often unrecognized In the last 10 years, I have prescribed the following treatment early ambulation and an intake of calcium equivalent to 3 or 4 glasses of nonfat milk a day in addition I

have advised the Albright Reifenstein regimen of cyclic administration of androgens and estrogens I agree with Henneman and Wollach[12] that by administration of estrogen (orally as Premarin or stilbesterol or as intra muscular injections of estradiol benzoate or valerate) it is possible to pre vent further fractures and arrest the progress of the disorder Thus it appears that steroid hormones are preventive, but not curative treatment for osteoporosis.

DR. SISSONS Someone asks the following question "Is it possible to tell from the histologic appearance of bone whether bone formation is being inhibited by corticosteroid?"

I hope I have been able to show you that it is, in fact possible to infer from the histologic appearance whether or not bone formation has been inhibited if the change is sufficiently marked As with any estimate this must involve some comparison with normal In the cases of Cushing s syndrome that I described to you, the changes were so severe that there was virtually no bone formation that could be identified in any of the material studied.

It is, of course, not possible to decide merely from the histologic appearance whether the effect on bone is a direct or an indirect effect of the corticosteroid hormone By looking at the bone we can say only how active certain processes are and not what particular agent is producing them

DR. NORDIN I have three questions here The first one is whether I have seen x ray changes or biopsy changes in people on high calcium intake The answer to that is no I have not done any repeat biopsies after treat ment, and I have not seen x-ray changes.

I think the explanation for that is a twofold one I have been doing this only for a year and a half and I would have been surprised to see any x ray changes yet, particularly because I am not measuring bone density

This brings us on to another question which says "Why does menopausal and senile osteoporosis manifest itself strongly chiefly in the spine?"

I think there is no doubt, or at least there is no doubt in my mind, that the reason for this is that the spine is predominantly trabecular bone Just as in the animal experiments on low calcium diet, particularly the one that Albright and Bauer and Aub did of which I showed you a summary it was the trabecular bone which was lost. I think most evidence would indicate that it is the trabecular bone that presents the largest surface to the tissue fluid is in the most immediate exchange with the minerals in the tissue fluid and that if negative balance develops, it is inevitable that the trabecular bone is predominantly affected and is taken out first.

This trabecular bone is at the ends of the long bones It was lost, you noticed, in Dr Whedon s study on the immobilization type of osteoporosis the trabecular bone goes first In low calcium experiments on sheep which have been done at the Rowett Research Institute a severe osteoporosis was produced in sheep on low calcium diets, and the spine was predomi-

nantly affected. The greatest difference between the controls and the experimental animals was in the weight of the vertebral bodies. This is not osteomalacia The bones have a normal ash content, but the vertebral bodies have lost bone and it is for this very reason, I think, that I have not picked up any change on therapy because I would expect to get a change only in the density of trabecular bone at the ends of long bones or in the spine and I have not so far been able to develop any satisfactory method of measuring the radiologic bone density so I have kept off it.

The final question is "Cannot the late rise in calcium balance in your four cases be explained on the basis of a natural remission of the disease which so frequently occurs in senile osteoporosis?"

In the first place it was not a late rise in four cases but in one after vitamin D therapy In the other three I gave them only a high calcium intake and they went into positive balance The question, therefore is based on a misunderstanding of what I said

With regard to the second part of the question as to a natural remission in the disease which so frequently occurs, I don t believe there is such a thing as a natural remission in this condition. I think it has a natural history I think the patient ultimately when he has lost enough bone, comes into calcium balance and ultimately adapts, and that is why Dr Urist s cases, who are old, have low urine calciums, whereas the youngest of my cases have got the highest urine calciums.

It is not a question of some osteoporotics, one group having a high urine calcium and one group having a low urine calcium it is a question of people ultimately adapting ultimately their urine calcium does fall, and ultimately they come into balance That is why some of the cases you see are in positive balance and some are still in negative balance

DR. URIST I will sum up my remarks by stating that the essential difference between Dr Nordin s position and mine is that Nordin regards negative calcium balance as the cause of osteoporosis I regard the osteoporosis as an endocrine disorder (of unknown etiology) which is the *cause* of the negative calcium balance It seems an oversimplification to regard the bone picture of calcium deficiency that is seen in a young growing animal as the same as osteoporosis in man low calcium diet produces mobilization of what Aub and his associates described as the "readily available calcium stores " These observations were made on kittens fed a diet free of milk We[14] observed osteoporosis (low bone mass) in young growing rats weaned to a diet barely adequate in calcium content (0.2 per cent) but the bone mass rapidly increased as soon as the animals reached maturity and attained the plateau of their growth curves Thus, after the demands of growth were satisfied the animal was able to make up the previous deficit in calcium even on a low calcium diet We[15] observed osteoporosis combined with rickets and osteitis fibrosa, in rats weaned to a diet low in both calcium and vitamin D this diet contained

only 0 06 per cent calcium and also produced stunting of growth We have not yet, however produced an experimental animal with osteoporosis because to qualify as a condition comparable to osteoporosis in man, it should develop in adult life and it should present thinning of the cortex of the vertebra expanded intervertebral discs, and spontaneous fractures Furthermore to establish the diagnosis of osteoporosis, the typical change should be in cortical bone it should be fully calcified and show resorption cavities but no osteoclasts *In the literature on biopsy the changes in* cortical bone which I regard as a characteristic feature of this disease seem not to have been considered

CHAIRMAN REIFENSTEIN I think the time has come for us to terminate this session Before I do I would like to thank the essayists and discussants for all they have done not only to mark out thoroughly the area in which we have some information, but also those areas in which we do not, and in which we are desperately in need of more investigation and study

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Part II

Dynamics of Calcium Metabolism

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Chemical Dynamics of Bone Mineral*

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Introduction

The chemical and physical nature of bone mineral is a topic long plagued by controversy On the one hand are the chemists with *their* data on the other hand are the structural people probing derivatives of bone or geologic specimens with x rays, and it has been rare indeed that the two imperfect sets of data have led to the same conclusions. It seems fruitless here to review this long struggle Rather only the present state of affairs will be summarized particularly with respect to the directions of profitable research in the immediate future

For purposes of this discussion, then we shall assume that the bone mineral is a single solid phase having a structure characteristic of the mineral apatites We will recognize that the exact nature of the carbonate in bone is not yet settled and that some people prefer to use some special name for this mineral substance some name other than hydroxyapatite However it is named, it is bone mineral in which we are interested, and for this discussion, it will be termed hydroxyapatite

In Table 5-1 are summarized the results of calculations made in our laboratory and the general results of the x ray investigations of many laboratories. From the accepted values for various ions in normal serum corrected for activity coefficients, serum ion products were compared with solubility products of a number of compounds which might be expected to occur in bone Of the lot, only one of these substances would be expected to form in body fluids for if the others did form they would dis

* This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester New York.

Table 5-1

A COMPARISON OF CHEMICAL AND X RAY EVIDENCE ON THE NATURE OF BONE MINERAL

Mineral	K_{sp}	*X-ray*
$CaCO_3$	−	−
$MgCO_3$	−	−
$NaHCO_3$	−	−
CaHPO	−	−
Apatite	+	+

solve On chemical grounds, we cannot expect to find any of these substances other than hydroxyapatite. Hydroxyapatite does not exhibit a fixed solubility product. It exhibits, rather a variable solubility a range part of which is well below the calcium phosphorus ion products found in normal body fluids Therefore on chemical grounds only hydroxyapatite can be expected to be present and stable.

Fortunately for all concerned, hydroxyapatite is what is found by x-ray diffraction Despite many searches, none of the other substances has ever been shown to be present by x ray investigation

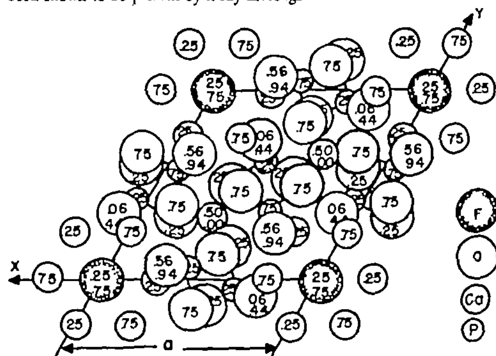


FIG. 5 1 A projection (on the 001 plane) of the atomic arrangement in fluor apatite according to Carlström In hydroxyapatite the positions occupied by fluorine are replaced by hydroxyl groups. (*From W F Neuman and M W Neuman "Chemical Dynamics of Bone Mineral" University of Chicago Press Chicago* 1958)

In a qualitative sense then there seems to be pretty solid evidence that the mineral substance of bone is the basic calcium phosphate known as apatite

Crystal Formation

How do crystals of hydroxyapatite form? If we try to produce hydroxy apatite in vitro we find that precipitation of hydroxyapatite as such does not occur directly[1] This is not surprising because the apatite structure has as its smallest repeating unit a total of 18 ions, shown in Fig 5 1 as Carlström[2] has pictured it. Obviously these ions could not all collide simultaneously in solution. The formation of hydroxyapatite must involve an intermediate transitory stage

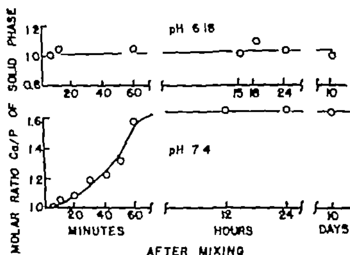


FIG. 5-2. Data showing the spontaneous conversion of an initial precipitate having a Ca/P ratio of unity to that of apatite (1 66) at physiologic pH Note that at lower pH 6.18 the initial precipitate is stable and its Ca/P ratio remains unity In such a precipitate, crystals of $CaHPO_4 \cdot 2H_2O$ are seen

Figure 5 2 shows the transition of initial precipitates of calcium and phosphate to hydroxyapatite The conversion is shown in terms of the calcium/phosphorus ratio This chemical evidence has been confirmed by direct x ray analyses of the solid at different times after precipitation

We know then that to form hydroxyapatite we must first go through a stage which appears to be secondary calcium phosphate At least the Ca/P ratio is close to unity in the very first solid formed Unfortunately the body fluids are not saturated with respect to secondary calcium phosphate[1]

It appears unlikely therefore that a direct precipitation of calcium phosphate is the mechanism by which bone mineral is formed in the body

There are two likely ways in which bone mineral might be formed It

might be formed through some booster mechanism whereby the produ the ions was increased locally This is similar to the old suggestio Robison[3] of a local production of phosphate ions by the hydrolysis c ester Or it might, on the other hand, involve some kind of catalytic pro in which an organic material served to collect the ions in a form promo the formation of hydroxyapatite.[4]

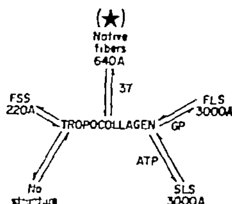


FIG 5 3 A diagrammatic representation of the specificity of crystal induction by collagen. In this instance a single preparation of tropocollagen obtained by the acid extraction of connective tissue was caused to crystallize in five different forms as well as the noncrystalline form. The various crystalline aggregates were then tested for their ability to induce crystal formation in a calcium phosphate system. Only those fibers having the normal x ray spacing characteristic of native collagen were able to nucleate. This is indicated by a star on the diagram.

Actually we do not know whic these two general possibilities is *and applicable, booster or catal* There are three principal areas of search exploration at the present t The first is based on the idea mucopolysaccharides in some way c lytically induce the aggregation of i to form the initial crystal. Sobel and collaborators are active in this fie A second line of research effort been directed primarily by Cartier a his group in Paris.[6] This is based the general idea that a special ene transfer is required Perhaps, in C tier's view this energy requiremen met by the transphosphorylation some matrix substance using A (adenosine triphosphate) as the pov compound. This phosphorylated r trix, then, presumably acts as the n cleating site for initiating the first b ginning bone crystal. A third line research has been directed toward t role of collagen. This has received *tention in our laboratory*[1, 4, 7, 8] a more recently at Massachusetts Ins tute of Technology by Glimcher and collaborators.[9]

We already know that collagen *can* serve as a nucleating center causi crystals to form from solutions which, in the absence of collagen, a stable for indefinite periods of time [10] Collagen *does* nucleate, but the are many unresolved problems. There seems to be evidence both fro the Massachusetts Institute of Technology group and our own that muc polysaccharides in this system actually inhibit rather than aid cryst nucleation There is considerable question whether the various collage are equally able to nucleate In our hands, the more purified the collage the better it acts as a nucleating center On the other hand there is co siderable question as to whether the collagen, as originally secreted by th

cell bears much structural relationship to the final product obtained from a demineralized, maltreated piece of bone or connective tissue The reason that nucleation by collagen seems like the best bet is the exquisite specificity of the process, shown in Fig 5 3

Another of the more interesting side lights in this general problem is Glimcher s finding[11] that there seems to be no preferential orientation of the small crystallites in the early stages of growth in vitro This is in sharp contrast to the fact that the fully formed crystals of bone appear to be well oriented as shown by Robinson and his co-workers in this country [12] by the Karolinska Institutet group in Sweden [13] and by many others, as well as hints in the older literature from x ray evidence This represents a sharp discontinuity between the initial crystal the initial aggregate, and the final crystal which grows Whether this represents the growth of only certain small seeds which have preferential orientation such that certain oriented crystal faces grow more rapidly than others or whether some totally unexpected phenomenon is operating here is not at all settled From the use of isotopes, we are well aware that the small crystals in forming bone appear to be undergoing very rapid recrystallization in the classic sense that is, an actual dissolution and re formation of the crystals This would be compatible with the view that the unoriented seeds tend to dis solve and the properly oriented seeds grow at their expense

In summary then, we are not sure whether data obtained in vitro really apply to the physiologic situation Much of the work in vitro has been done at 25 C Very little has been done at 37°C Current investigations on the mechanism of calcification have not yet pinpointed the area in which we should concentrate our search We know that the old schemes suggested by the Robison hypothesis had best be left to history while we look for new possibilities which might be operative In other words, we know now that we are much more ignorant than we thought we were when Robison s scheme was first suggested. This represents some progress An awareness of ignorance is prerequisite to intellectual growth.

Crystal Organic Interrelations

Let us ignore the problem of crystal initiation and start with a fully formed bone crystal We find that the habit of the crystal is not truly established Robinson and Watson first described the bone crystal as a tiny tablet.[14] Engström s group at the Karolinska Institutet with a limited number of specimens described a rod [15] It is fair to say that Robinson has now also described rods and Engström and co-workers have described tablets To add further to the confusion, workers at Argonne have de scribed a needle [16] From studies in vitro it is known that the habit and size of the apatite crystal are largely dependent on the composition of the fluid in which it is formed On this basis, one wonders whether there is

any ground for an argument here whether or not the crystalline habit of apatite might not be quite variable, and whether in fact all three forms and perhaps others do exist in bone

Whether it is a tablet, needle or rod, all workers agree that the size of the crystals is approximately 50 to 70 A in thickness and a few hundred angstroms in length No direct surface area measurements of bone mineral are available Examination of specimens isolated by mild procedures lead us to think that the specific surface area of bone mineral is of the order of 200 or 300 sq m/Gm This specific surface area seems enormous, but it does correspond rather closely to the dimensions described from electron microscopy and low angle x ray scatter—bits and pieces of the order of a hundred angstroms or so in dimensions

All morphologists are agreed that there is a close relation between the collagen fibers and the crystals In fact, all agree that the C axes of the crystals parallel the longitudinal axes of the collagen fibers to a remarkable degree

There is some uncertainty still but the conviction is growing that crystals *are on, in, around, and between the collagen fibers* This is more or less to be expected on chemical and crystallographic grounds The presence of a few crystals will induce the formation of others by dislocation of small aggregates which then can grow spontaneously

Crystal Growth

We can be quite sure that there is a process of crystal growth involved in the maturation of bone After all, crystals cannot suddenly appear *de novo* They must start small and become larger An important question however is whether they all grow to a certain size and then stop or whether crystal growth is an ever-continuing process The importance of this will become more clear as we proceed.

Electron microscopic studies by Watson of human bone specimens taken from various age groups gave the impression that the size of the crystallite in bone is a function of the age of the person [17] The crystals in young bones seemed smaller than the crystals in senile bone. Even the collagen fibers themselves seem to be in a variable state of aggregation, having larger dimensions in very old bone These studies, however were very limited, and we can regard the conclusions as somewhat tentative

There are a number of other kinds of evidence that could be interpreted as suggesting a gradual increase in crystal size. One of these has come from a study of the escape of the noble gases thoron and radon from skeletally deposited radium and mesothorium Norris[18] and later Mays have studied this problem.[19] It has been found that the fraction of radon which escapes and appears in the exhaled air decreases with increasing skeletal residence

time of the parent radioisotope Mays has shown how this is explained in terms of crystal size His explanation is illustrated in Fig 5-4

Actually this kind of evidence is not truly definitive It is another indicator that the density of bone structures increases with increasing time It can support the view either that the crystals are increasing *in their dimensions or* that there are increased numbers of crystals.

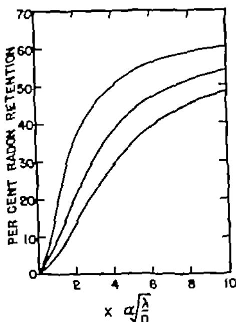


FIG 5-4 A diagrammatic representation of the percentage of radon retained by the skeleton as a function of crystal size These calculations were made by Mays and *X* the unit on the abscissa, gives a slightly different relationship depending upon whether the crystal is assumed to be a sphere, a cylinder or a tablet. Whatever the true shape of the bone crystal it is clear that radon retention by the skeleton would be expected to increase with increasing crystal size. (*From Atomic Energy Rept* COO 216 1958 *by permission of C W Mays Jr University of Utah*)

Perhaps the most decisive evidence is derived from studies of the water content of dental and bony structures It was Deakins who first showed that the composition of bone is best represented on a volume basis [20] The reason for this is now obvious As one puts into a given volume of structure varying proportions of water organic and inorganic materials the density is bound to vary Expressed on a weight basis *therefore, composition is some*what misleading On a volume basis, one gets a true picture of the relative proportions of the three constituents Deakins was able to show in enamel that the organic content of the enamel remained relatively constant and that with increasing age the mineral constituents increased at the expense of the water volume These two were directly related—a displacement of water by mineral More recently Robinson and Elliott have been able to show that the same rule applies to bone [21]

This kind of study then also supports the view that there is a gradually increasing degree of mineralization in a structural element as it ages It does not tell us whether the crystals themselves just grow larger or whether the formation of new crystals gradually fills up the total volume of bone until practically no water is left. From the physiologic standpoint, this question as to number or size of crystals may prove relatively unimportant but the gradual exclusion of water volume by increasing mineralization is of great importance

The Crystal as a System

We are reasonably sure from limited studies that unashed, unheated apatites, such as bone mineral, are extremely imperfect in their crystalline structure Apparently many voids and defects are present within the lattice This defect structure permits a slow diffusion of ions from the surface into the interior a process first termed "recrystallization" but more properly termed "internal exchange" or "intracrystalline exchange" or "internal reconstruction" or "diffusion in a solid."

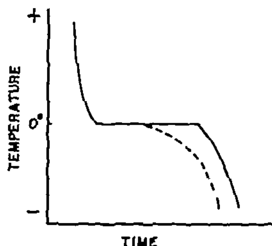


FIG. 5 5 A typical cooling curve of water in the absence and presence of hydroxyapatite crystals. The solid curve is that obtained when only free water is in the system. The plateau at 0 C indicates ice formation. In the presence of crystals, the cooling curve is identical with that of free water down to 0 C. The plateau, however is not nearly as long and the temperature gradually falls as water bound in the hydration shell of the apatite crystals gradually freezes.

At the surface the crystals bind an enormous quantity of water and are highly hydrated. The hydration phenomenon was discovered in attempts to isolate the solid phase free from bulk solution purely for analytic reasons Thus the first evidence of hydration was based on the fact that a large quantity of water could not be centrifuged away from apatite crystals.[22] Such data are open to question As the crystals get closer and closer together during the process of centrifugation, one can logically reason that capillaries, or the equivalent of capillaries, would be formed and that much of the water might be held by simple capillarity There were reasons for believing that capillarity was *not* operative in this case [22] but some additional evidence was needed This was obtained by a different experimental system.

The system is rather complicated in operation, but in principle it is very simple One sets up a container from which heat can be withdrawn at a constant rate As heat is withdrawn, the temperature falls. If water is in the system the temperature will fall until ice begins to form at zero degrees At this point the temperature cannot fall until the heat of fusion has been satisfied, and the amount of heat or in this case the length of time which the system remains at zero degrees is a measure of the free water in the system. Figure 5 5 shows what happens when the same volume

of water is placed in a container in the presence of some apatite crystals. The amount of free water is sharply reduced and the system behaves as though it contained a volume of water which had a varying salt content, freezing at various temperatures below the ordinary freezing point of water Now in this free suspension of crystals there could be no capillaries between crystals The volume of water held by the crystals in this experiment was somewhat greater than that observed with the centrifugation technique This is not unexpected because the experiments were done at different temperatures

It has also been possible to devise isotopic techniques which permit partial analysis of the composition of this hydration shell This involves a kinetic analysis of exchange data. The analysis itself may be subject to errors of interpretation However if correct, the concentration of calcium and phosphate in the hydration shell exceeds that in the surrounding solution by a factor approaching a thousand In other words, the ionic strength of the hydration shell is much greater than that of the surrounding solution under ordinary circumstances.

Supporting this interpretation is the observation that many many ions tend to concentrate at the solution–crystal interface If the ionic strength at the interface is high one can expect many ions to concentrate at that interface One can also predict the kind of ions which will concentrate at such an interface The explanation is quite simple Consider an aqueous suspension of apatite crystals in a beaker If one introduces a multivalent cation into this system, it will diffuse from the aqueous phase into the hydration shell around the crystal, and the net transfer of ion will not stop until the chemical activity of this ion is the same in hydration shell as it is in the bulk solution. The chemical activity however is not equivalent to concentration The theory of interionic attraction tells us that, in a solution of ionic strength such as that in the hydration shell immediately surrounding the crystal the effectiveness of an ion is greatly reduced by the presence of neighboring charges and fields In other words, for the *effective* concentration to be equal in the hydration shell to that of the bulk solution, its actual concentration must be much greater Interionic attraction theory also tells us that the higher the valence of an ion the more likely it is to concentrate at the interface This is, in a general sense exactly what is observed. Monovalent cations such as potassium and sodium do not tend to concentrate to any great degree in a hydration jacket Bivalent and more particularly the trivalent cations concentrate to a marked degree

From studies of the concentration of potassium ion at the interface the average ionic strength of the hydration shell has been estimated to be about *1.5 M* It would be naive to assume that the ionic strength of the entire hydration shell was 1.5 Rather it is more reasonable to presume that the ionic strength at the outer edge of hydration shell is quite low similar to

the surrounding bulk solution, and higher near the crystal surface In any event, the effect of this strong highly concentrated solution immediately surrounding the crystals is to cause all highly charged highly hydrated ions to concentrate at the crystal solution interface

One point must be stressed. *The interface as it is now understood is the basis for all chemical and physical properties of the bone mineral* It is a pity we have such fragmentary information and such a crude conceptual understanding If the crystal dissolves there is a net transfer of ions through this hydration shell to the bulk solution Crystal growth is merely a net transfer in the other direction Even in a static system, there is a continuous exchange of ions back and forth from the solution into the hydration shell to the surface and so on. In other words, for every ion which moves in one direction or the other in a net sense, hundreds or thousands of ions are bouncing back and forth at all times. The same underlying process then, applies to dissolution crystal growth, and classic exchange.

The process of ionic transfer to and from the crystal surface to the concentrated milieu, that is, the hydration shell, is responsible for the puzzling and variable stoichiometry of the apatite minerals Because the crystal surface is so tremendously large and because the requirements of space and charge are not nearly so rigorous in this location as in the interior of a crystalline lattice a wide variety of ion substitutions can occur For this reason the composition of hydroxyapatite mirrors, in a very complicated way the composition of its fluid environment. If the environment contains sodium, so does the solid phase If it contains carbonate so does the solid phase and so on. As a result of this extensive ion exchange process, we find that bone mineral is not a pure hydroxyapatite by any means Rather it contains many of the ions found in the extracellular fluids—sodium, carbonate citrate magnesium and traces of fluoride Because of the complicated and variable composition of this mineral substance its solubility is not fixed but is variable It is a well-established truism of physical chemistry that any substance exhibiting variable composition cannot exhibit a fixed solubility

While this gives an explanation for some of the peculiar properties of the hydroxyapatite system it is a most unsatisfactory explanation for it does not permit us to predict accurately the behavior of the apatite in a given instance nor does it enable us to design experiments in the laboratory in which only a single variable is operative

The Water of Bone

The state of water in bone is one of the most perplexing problems of all. Crystals suspended in free solution appear to take up a surprising volume of water greater than twice that of the crystals themselves. On this basis

one would expect that the water content of any calcified structure would never fall below a minimum of 40 or 50 per cent Obviously this is not the case. The water content of mature and compact bone may be as low as 10 per cent Obviously there is not sufficient water present to hydrate fully all the crystals present.

The underlying reasons for such a remarkable dehydration of bone are not clear at the present time With our limited information it seems reasonable to attribute this phenomenon to the supersaturated condition of the body fluids with respect to calcium and phosphate However the supersaturation of the body fluids is in itself not a simple matter and is poorly understood It is well established that serum as it is drawn from the vein is highly supersaturated [1] It is also reasonable to presume that the extracellular fluids are similar in composition to serum However the solubility of apatite mineral and, therefore, of bone mineral is variable and dependent on the composition of its surrounding medium Since recent studies have shown that the metabolic actions of bone cells have a local effect on the composition of the fluids bathing the bone crystals [23] one cannot be certain that the fluids directly in contact with the bone mineral crystals are as supersaturated as one might infer from an analysis of serum. From these considerations, one is led to the tentative conclusion that the crystals are under tremendous stimulus to grow until they can grow no more. This stimulus results from the supersaturation of the body fluids Only by the active resistance on the part of cells by their secretion of acids and chelators such as citrate can this stimulus be overcome

Maturation of Osteons

A naive comparison of the growth of bone crystals with the precipitation of calcium phosphates from a solution would lead one to expect that the mineralization process would be one of very short duration As soon as the crystal seeds started to form, the supersaturation of the extracellular fluids would make precipitation occur almost immediately One might expect to find only unmineralized or fully mineralized bone Obviously this is not observed, and it is not the case one *should* expect. In vivo the crystals are forming in a fixed volume. As the crystals grow they must grow closer and closer together the hydration shells must ultimately interact, and the diffusion of ions through the highly concentrated jackets containing ions which are partially labile and partially fixed structurally becomes slower and slower As the crystal faces approach each other the restriction of charge and volume gradually will slow diffusion to a point where it is stopped almost completely

On this basis, one should expect to find, in bone areas which have not mineralized and areas which, once started would go *almost* to full mineralization As these osteons become more and more mineralized the rate of

further mineralization becomes slower and slower This is, indeed, observed and the latest microradiographic studies show that the bulk of the osteons which one sees in ordinary bone cross section are almost but not fully mineralized, varying from 90 to 100 per cent of full mineralization [24] Very few if any are found outside this range The reason of course, is that the time spent in getting to 90 per cent full mineralization is very brief, while the time spent in getting from 90 to 100 per cent full mineralization is very very long.

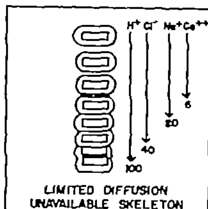


FIG. 5-6 A diagrammatic representation of the limited diffusion of ions in the skeleton. Here the distances between the crystals are exaggerated to show that, in closely packed areas, the distances become so minute that the hydration shells of adjacent crystals have fused. Under these circumstances only hydrogen ions can penetrate freely Unhydrated monovalent ions such as chloride can of course penetrate fairly well, while hydrated cations are very restricted. The numbers in the graph represent approximate percentages of the ions present in an adult skeleton which will undergo ready exchange.

This phenomenon of supersaturation which leads to the general continuing maturation and mineralization of each bony element may be responsible for the apparent irreversibility of ionic interchanges in the skeleton. If one introduces a radioisotope into the circulation, it can rapidly diffuse into the areas surrounding bone crystals. In areas where the osteoblasts are laying down new matrix and new crystals are forming, the free diffusion of ions is almost complete. Within minutes or hours however the crystals have grown very rapidly to perhaps 90 per cent of full mineralization. At this point, the diffusion of ions is severely limited. As a result, ions which could enter freely and in a reversible fashion have become more or less trapped and cannot freely diffuse out again. This process, extremely important where there is new mineral formation is also important but to a lesser degree in *all* areas of bone. An ion may find it difficult to diffuse in an osteon that has been established for some time but it will find it even more difficult to diffuse out.

The general continuing maturation of all bony elements "explains" the apparent irreversibility of what otherwise would be physicochemically reversible processes.

The concept of a limited diffusion of ions is important. It is a relatively new concept, still in an embryonic form There is reason to question whether it will survive. That it will grow robust and strong as our information increases is suggested by recent studies on the changing reactivity of the skeleton at various ages, as measured by radioisotopes.

For years there has been talk of "labile bone" and "stable bone" of "available bone" and "unavailable bone" These terms are proving to be only relative the degree of availability is variable depending on how it is measured. If deuterium is used the entire skeleton is "available" If radiosodium is used less is available and the age of the animal becomes important

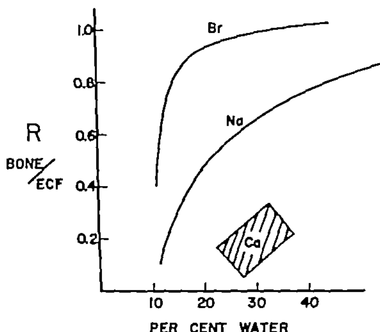


FIG 5 7 The relationship between skeletal exchange and the water content of the skeleton itself. On the ordinate is plotted *R*, the ratio of the radioactivity in bone relative to the radioactivity in extracellular fluids. A ratio of unity indicates complete exchange.

These relationships are diagrammatically represented in Fig 5-6 and are also more precisely shown in the data of Forbes[25] given in Fig 5 7 The tiny unhydrated deuteron like hydrogen, can diffuse freely through the entire skeleton Larger monovalent ions experience more difficulty Their diffusion is blocked by the proximity of charges and ions in the more fully mineralized areas of bone Monovalent ions which are highly hydrated and therefore occupy a larger volume have even greater difficulty diffusing throughout the skeleton Such an ion is sodium Highly hydrated multivalent cations such as calcium are very restricted in their diffusion through the skeleton. Only in the relatively newly formed bone can calcium diffuse at all efficiently As a consequence only a small fraction of the calcium in the skeleton is readily exchangeable It must be admitted that the *exact* percentage is not known with any certainty but there is no doubt that it is extremely small

Perhaps the most convincing argument in favor of the concept of limited diffusion is based on calculation of the space between crystals in the adult

skeleton Such space must be occupied by either organic matter or water With conservative assumptions, the intracrystalline spaces can be calculated, and these calculations indicate the size to be of the order of a few angstroms It is almost a physical certainty that, under such circumstances, the free diffusion of ions is virtually impossible

Summary and Conclusions

In summary the structure of the bone mineral is fairly well understood but several very important problems remain unresolved, for example, the exact nature of the carbonate in bone The mechanisms by which new crystals are formed in bone and cartilage are not understood. Perhaps the most promising developments in this area involve the discovery that collagen fibers can nucleate crystal formation at concentrations below the point of spontaneous precipitation. There seems to be a considerable body of evidence indicating the degree of mineralization with increasing age of the skeletal unit, but it is not yet established whether this involves a continued growth of individual crystals or the formation of new ones. The nature of the water of bone is only partially understood. It is, however clear that the skeleton as a whole is quite dehydrated This dehydrated state may be responsible for its inertness and lack of physiologic reactivity The gradual loss of water as skeletal units age may be responsible for the apparent irreversibility of the many ionic interchanges undergone by the skeletal mineral.

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6

Kinetics of Calcium and Strontium Metabolism in Man

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Introduction

Bone is constantly being formed and resorbed. When the skelet not change its shape or size as in the normal adult, the over-a of formation and resorption balance each other If the skeletal m creases, as in children, formation dominates, and if it decreases as age resorption dominates. *The balance between bone salt format* resorption is reflected by the difference between calcium intake a cretion, provided that the extraskeletal calcium content does not appreciably The extraskeletal calcium normally forms less than cent of the total body calcium. The body does not tolerate large c in the body fluid calcium concentration The calcium balance is t general negative when resorption dominates positive when for

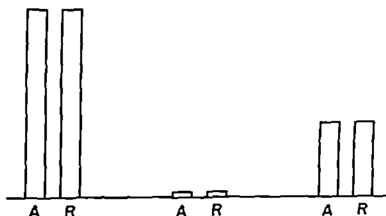


FIG. 6-1 Three clinical situations in which metabolic activity varies (l columns) but bone formation (*A*) and resorption (*R*) proceed at eq

inates, and zero when formation and resorption proceed at equal
s

Under various pathologic conditions bone resorption dominates over
nation in the adult individual the skeleton loses bone In experimental
nals such a condition is easy to assess—for example by serial sampling
one. In man advanced loss of bone may be detected with x ray how
 x ray techniques generally seem to be insensitive to even a 50 per
t loss in body calcium

Calcium balance or x ray techniques do not reflect the metabolic level
ever This obvious but important fact is illustrated simply in Fig. 6-1
re may be three different clinical situations in which there are great
ations in metabolic activity but in each of which bone formation and
orption proceed at equal rates so that the calcium balance is zero It is
e that considerable hope may be attached to isotope techniques, in much
same way as they are used for studies of thyroid metabolism not only
research but also as a routine diagnostic procedure This chapter will
nonstrate that isotope techniques may be used in man for studies of the
e of bone formation in the entire skeleton and in localized skeletal
ons

Isotope Studies of Skeletal Metabolism

Normal Conditions. Following rapid intravenous injection of a single
e of radioactive calcium or strontium the blood radioactivity rapidly

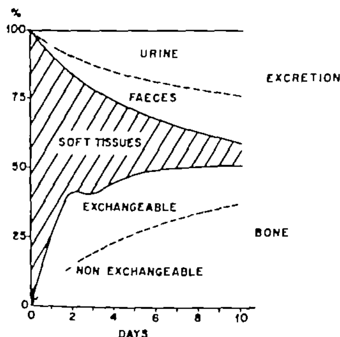


Fig 6-2. Distribution of Ca^{47} after single intravenous injection.

drops (every fourth calcium atom leaves the blood per minute) while the radioactivity in the extravascular fluid reaches a maximum and then drops in parallel with the blood radioactivity Some radioactive calcium is excreted via kidneys and intestines, and with passing time it is found that a large part of the injected radioactive calcium has accumulated in bone (Fig 6-2) especially in areas of active bone formation This rapid accumulation of radioactive calcium in the skeleton is partly due to exchange of skeletal calcium with circulating calcium The soft tissue radioactive calcium thus mixes with the exchangeable calcium of the skeleton In adult man this latter fraction makes up for about 0.2 per cent of the total skeletal calcium By far the greater part of the skeletal calcium is unavailable for exchange with circulating calcium Radioactive calcium in the soft tissues can therefore be incorporated in this part of the skeleton only by formation of new bone salt. With increasing time following injection of radioactive calcium the activity in the nonexchangeable part of the skeleton therefore rises, while the activity in the exchangeable part drops in parallel with the soft tissue radioactive calcium. Practically all the radioactive calcium retained in the body will therefore ultimately be located in the nonexchangeable skeletal calcium

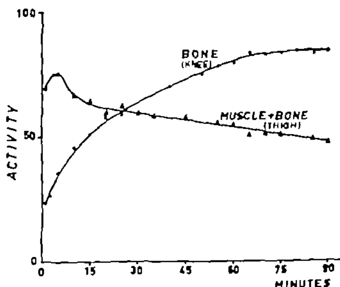


FIG. 6-3 Body surface counting rates after single intravenous injection of Sr^{85}

A comparison of the body retention excretion, and blood activities following injection of radioactive calcium permits a calculation of (1) the amount of exchangeable calcium in the body and (2) the rate at which the nonexchangeable bone salt is formed in the skeleton, i e the accretion rate. In the normal adult new bone salt is formed at a rate of about 0.5 Gm calcium per day and a corresponding amount is resorbed The adult skeleton contains between 1 000 and 1,500 Gm calcium and is thus re

newed at a rate of about 0 05 per cent per day In the newborn the corresponding value is 1 per cent or higher However some caution must be exercised in the interpretation of these turnover values, because the rate of renewal varies considerably from area to area in the skeleton In the trabecular bone in the metaphyses in the vertebrae and in the flat bones of the pelvis, the rate of renewal is considerably higher than for example in the cortex of the long bones.

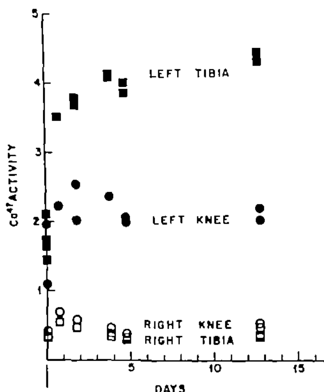


FIG. 6-4 A 54-year-old woman had had a localized Paget lesion in her left tibia for at least 15 years. There was no evidence of skeletal involvement other than her left tibia. The patient received 60 μc Ca^{47} by intravenous injection The absolute counting rates shown in the graph indicate that the metabolic rate in the pagetic bone was considerably higher than normal. (*From R. C Extermann ed Radioisotopes in Scientific Research Pergamon Press London* 1958 *vol* 4 *p* 232.)

Use of γ-emitting isotopes of calcium (Ca^{47} half life 4 9 days) and strontium (Sr^{85} half-life 65 days) permit tracing the injected isotope by means of scintillation detectors located on the body surface Such studies in man show the rapid rise and fall in soft tissue activity and the slow rise in bone activity (Fig 6-3) Furthermore the external counting rate recorded over bone with rapid accretion is higher than over bone with slow accretion Under comparable geometrical conditions therefore the external counting rate ratio is roughly equal to the accretion rate ratio

The skeleton does not distinguish between calcium and strontium (in

trace amounts) The results obtained from external counting over variou lesions following injection of Ca^{47} or Sr^{85} will therefore be discussed belo without distinction between the two isotopes.

Pathologic Conditions. In our laboratory we are at present particularl interested in the interpretation of external tracings obtained following ir jection of Ca^{47} or Sr^{85} *Such studies in some cases of Paget s disease* demor strate the correlation between evidence obtained from conventional mo phologic techniques, measurements of blood and excreta activities, an external radioactivity tracings. Pagetic bone tissue is rapidly being rebui as evidenced by microscopic examination Following injection of Ca^{47} to woman with a Paget lesion in her left tibia, the external counting rat recorded over the lesion was several times higher than over a correspondin location over her right normal tibia (Fig 6-4) Finally an analysis o isotope data from four different laboratories shows that the accretion rat in generalized Paget s disease is significantly higher than normal.

We believe that similar studies of less clear-cut types of bone diseas will be of diagnostic value This conclusion is based on studies in *hyper parathyroidism* in which the accretion rate has been found to be highe than normal, and in several cases of so-called *osteoporosis* in which th accretion rate does not always seem to be lower than normal The follow ing list summarizes the findings from studies in several laboratories

Calcium Accretion in Bone Disease

High
- Paget s disease
- Fracture
- Tumor
- Hyperparathyroidism
- Hyperthyroidism
- Vitamin D resistant rickets treated with massive doses of vitamin D

Normal
- Osteopenia of unknown origin
- Vitamin D resistant rickets
- Vitamin D deficiency rickets after treatment with vitamin D

Low
- Osteopenia of unknown origin
- Hypoparathyroidism
- Hypothyroidism
- Vitamin D deficiency rickets

Conditions with locally increased accretion rates are particularly easy to study with external counting techniques. Figure 6-5 shows the counting rate recorded with external counting of Sr^{85} over 2-month-old femur and

Osteopenia is a term introduced to cover the concept 'too little calcified bone' in Albright s sense

tibia *fractures* as compared with the counting rates recorded over the normal femur and tibia in the same individual As can be seen the counting rates over the fractures were five to eight times higher than normal This allows the conclusion that the accretion rate of bone salt in these fractures was about five to eight times higher than in normal bones This technique was applied to a series of patients with intracapsular femoral neck

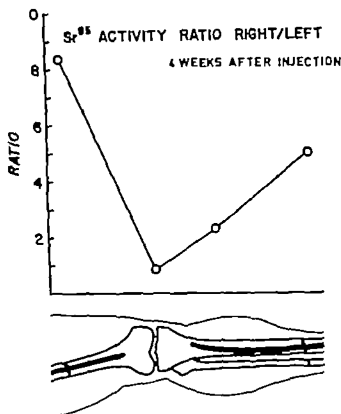


FIG. 6-5 A 17 year-old man who had suffered transverse fractures of his right femur and tibia in an automobile accident 2 months prior to the investigation. Both fractures were reduced and immobilized by means of intramedullary nails, which were inserted 6 weeks prior to the investigation. The patient received 20 μc Sr^{85} by intravenous injection (*From R C Extermann ed "Radioisotopes in Scientific Research" Pergamon Press London* 1958 *vol* 4 *p* 232.)

fractures. It was found that the accretion rate in the fractured neck rose to a maximum after about one to two months and then slowly dropped These results corresponded to similar findings in rats In a few of the patients, the ratios were found to be considerably higher than in the rest of the group In these patients it has subsequently been found that a pseudarthrosis of the neck has developed Similar findings have been made in established or threatening *pseudarthrosis* in femur and tibia fractures (Fig 6-6) The accretion rate in pseudarthrosis would therefore not be lower than normal but rather higher than normal. This somewhat surpris

ing finding would mean that a pseudarthrosis does not heal because increased resorption balances accretion

The finding that radioactive calcium introduced in the blood stream rapidly accumulates in a fracture callus suggests that the fracture callus cannot be regarded as a closed chemical factory as it has sometimes been described The calcium of the callus is derived from the body fluid. Calcium resorbed from the callus and from the fractured bone may of course ultimately reach the callus, but most of the resorbed calcium will mix with

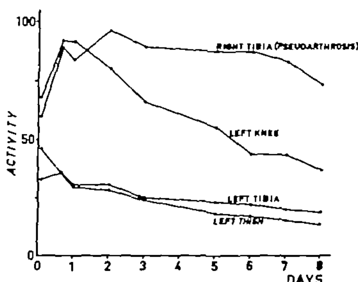


FIG. 6-6A. A 58-year-old man developed a pseudarthrosis following fracture of his left tibia, which was finally amputated. Nine days before amputation the patient received 47 μc Sr^{85} by intravenous injection and external counting was done as shown in the graph. It is seen that the isotope concentrates in the pseudarthrosis area.

the calcium of the body fluid It is an old observation that fracture causes loss of calcium from bone adjacent to a fracture This loss of calcium seems to be due to an increased resorption of calcium which most probably also mixes with the body fluid calcium.

Our investigations thus far indicate that most skeletal tumors cause an increased rate of bone formation even though tumor usually stimulates resorption of bone more than formation on x ray most bone tumors appear less dense than adjacent bone When radioactive strontium is given to a patient with cancer metastases to the skeleton, we invariably find a markedly increased uptake of radioactive strontium in the tumor areas (Fig. 6-7) Our findings suggest that metastases to the skeleton may be detected (and subsequently treated) before the lesions can be demonstrated

in radiographs It does not seem probable, however, that isotope techniques will be of use for differentiation between various types of skeletal tumor

Osteitis and *osteomyelitis* also cause a local increase in the accretion rate With passing time it is possible to demonstrate radiographic signs of subperiosteal formation of new bone Even before that time a local increase in injected Sr^{85} has been demonstrated with external counting

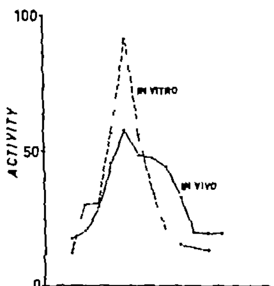




FIG 6-6B *Immediately before amputation, scanning of the pseudarthrosis area* was made with a narrowly collimated scintillation crystal. Immediately after amputation the tibia was dissected free from soft tissue and transversely sawed into 1 cm long pieces upon each of which counting was done. It is seen that there is good agreement between measurements in vivo and in vitro

Radiation Dosage. The studies reported in this communication have all been made with less than 1 μc of Ca^{47} or Sr^{85} and 0 1 μc of Ca^{45} per kilogram body weight These amounts give a mean weekly dosage of about 0 3 r or less, which corresponds to the maximum permissible dosage recommended by the International Committee for Radiation Protection

These recommendations apply to healthy persons within the reproductive age Many diagnostic procedures involve considerably higher dosage to the critical organ (for example, the conventional thyroid uptake study

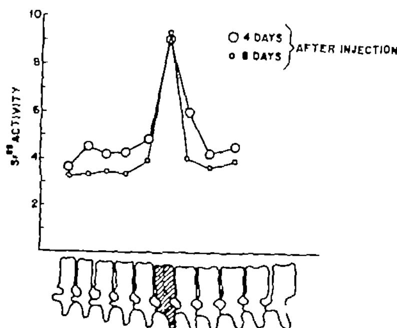


FIG. 6-7 A 68-year-old woman was operated upon for cancer of the left breast 1 year prior to isotope investigation. At the time of investigation x ray revealed a metastatic cancer in the 6 thoracic vertebral body The patient received 60 μc Sr^{85} by intramuscular injection. The 24-mm collimator was used. The activity is expressed in counts per second.

with I^{131}) or higher gonadal irradiation (for example, x ray urography or tomography of the lumbar spine)

Summary and Conclusions

There is considerable evidence from animal experiments that isotope techniques have been instrumental in obtaining a better insight into normal processes such as bone growth and repair and pathologic conditions such as vitamin D or protein deficiency It seems probable therefore that similar data in man will assume both research and clinical importance for clarification of the kinetics of calcium metabolism under normal and pathologic conditions

Acknowledgment

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7

Ground Substance and Calcification

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Earlier work on the ground substance of connective tissues[1] was extended to a study of the relations of this extracellular complex to calcification in cartilage teeth and bone [2-6] There appeared "to be a relationship of calcifiability of cartilage bone and dentin to the state of organization of the ground substance " [6] This conclusion was based on some simple observations made on sections of frozen-dried material stained with the periodate-leukofuchsin method In general, when calcium was being deposited or resorbed in bone or teeth, the ground substance of the affected regions stained more deeply by this method than in the fully calcified state or became water-soluble The increased stainability was attributed to the liberation of previously bound groups arising from depolymerization or disaggregation of ground substance components.

Further clarification and particularization of this concept came with the application of additional methods The first of these was developed by Joseph, Engel and Catchpole who used an electrometric technique to determine the colloidal charge at a given site.[7 10] By the use of salt bridges and calomel half cells, a circuit was made between the connective tissue under study and a distant body tissue Starting with isotonic saline junctions at the tissues, such a system will record zero or small base-line potential differences When at the experimental site saline is replaced by a more dilute salt solution (usually one-tenth isotonic) there is a change in the potential difference which is proportional to the colloidal charge density In effect, the difference of potential is also a measure of the concentration of water in the two regions By some technical and theoretical refinements, they were able to predict differences in ion concentrations in the extracellular ground substance

Underlying their work is the assumption that ground substance is a

two-phase system namely a water rich colloid-poor phase in thermodynamic equilibrium with a water poor colloid rich phase The ionic changes accompanying alterations in the relative proportion of each phase were conceived of as *determined* by the relative amount of each phase They predicted, for example that, when the water rich phase decreases and the water poor phase increases, there should be an increase in the sodium potassium, and total calcium The total calcium in particular could increase as much as threefold The observations and theory are important because they treat the whole of the ground substance regardless of its origin or composition as a thermodynamic system in which in general, the larger relatively immobile colloidal aggregates determine the concentration of the more mobile ions

By its very nature, such an analysis does not give specific information on the absolute dimensions of the two phases that is, their size and relations This information was supplied in part by Bondareff in his studies with the electron microscope of frozen-dried preparations of developing rat tail tendon [11] Bondareff found that in early developmental stages before collagen fibrils appear the intercellular material consists of a series of discontinuous vacuoles of low contrast enclosed in thin walls of higher contrast



Fig 7 1 Electron micrograph of tail tendon of 16-day-old fetus No fibrils are visible The ground substance appears vacuolated and the contents of the vacuoles are less dense than their walls (×10 000) (*Courtesy of W Bondareff Gerontologia* 1 222–233 1957)



FIG 7 2 Electron micrograph of tail tendon of 18-day-old fetus. Some fibrils are present. The ground substance appears as in Fig 7 1 (×4 000) (*Courtesy of W Bondareff Gerontologia* 1 222–233 1957)

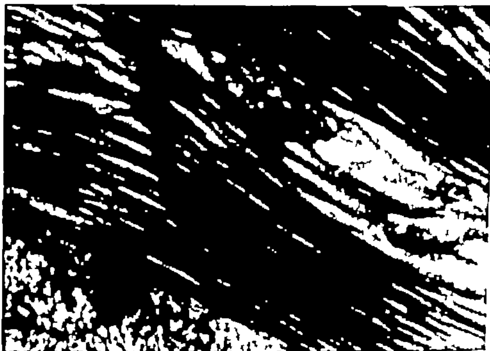


FIG 7 3 Electron micrograph of tail tendon of 10-day-old rat. Fibrils are numerous and well developed. The ground substance also appears similarly vacuolated as in Figs. 7 1 and 7 2. (×45 000) (*Courtesy of W Bondareff Gerontologia* 1 222–223 1957)

(Figs 7 1 to 7 3) He suggested that the vacuoles of low contrast were correlated with the water rich colloid poor phase, while their denser walls represented the water poor colloid-rich phase of Joseph Engel and Catch pole. These submicroscopic vacuoles persist in later stages, even after collagen fibrils appear

Bondareff made the further observation that the collagen fibrils appear first in the denser walls of the submicroscopic vacuoles and suggested that the fibrils arise extracellularly from unit collagen molecules, perhaps tropocollagen, of the ground substance He found no evidence for the intracel lular formation of fibrils or for the formation of fibrils in or on the surface of connective tissue cells These basic observations and suggestions on the structure and nature of the ground substance and the origin of collagen fibrils in it may be of importance also in the study of developing bone as I shall show later

Further work in this direction on the dynamics of ground substance would have been exceedingly difficult, if not impossible had it not been for a surprising development. Chase was able in a manner of speaking, to magnify the submicroscopic vacuoles to the point where their image be came visible with the light microscope [12] The choice of the preparation was

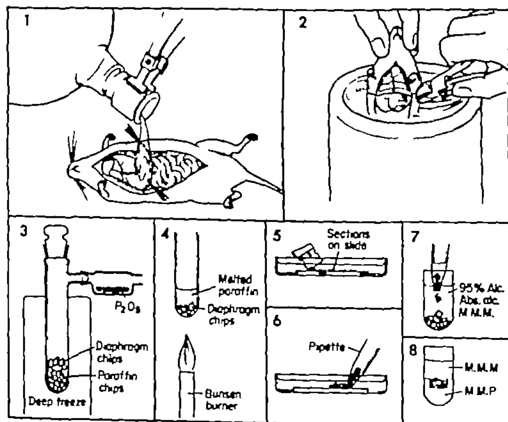


FIG 7-4 Diagram of the method of preparation of the mouse diaphragm for study of the distribution of ferrocyanide in the ground substance of the con nective tissue.

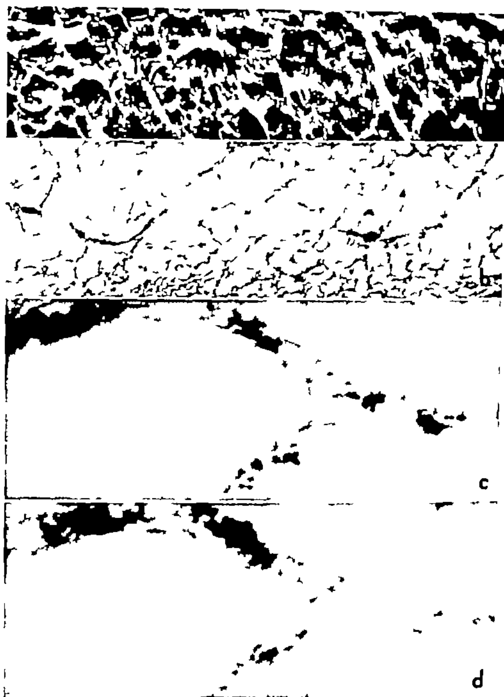


FIG. 7 5 (*a*) Photomicrograph of a section of the diaphragm of a young adult mouse, oriented transversely through the muscle fibers, stained with hematoxylin and eosin, for orientation purposes. (×300) (*b*) Photomicrograph of section of mouse diaphragm, stained only for ferrocyanide, which had been injected intravenously earlier The ferrocyanide (black in the print) is distributed discontinously in the ground substance of the connective tissue between the muscle fibers. (×300) (*c*) and (*d*) Higher magnification of a portion of a preparation similar to that in *b* at two different focal planes to show the small unit droplets which occur in clusters (×300) (*Courtesy of W H Chase A M.A Arch Path* 67 525–532, 1959)

different and was dictated by the purely technical factor of avoiding the formation of ice crystals during the freezing The procedure is as follows Mice are injected slowly with a large dose of sodium ferrocyanide Directly after completion of the injection, the mouse is bled and the diaphragm is exposed without cutting the muscle fibers and stretched. It is frozen very rapidly by pouring over it a large volume of propane at about −175°C (Fig. 7-4 1) and the whole animal is immersed in liquid nitrogen Pieces of the frozen diaphragm are broken off in liquid nitrogen (Fig 7-4 2) and dried in a vacuum at about −35°C (Fig 7-4 3) They are then infiltrated in vacuo with preevacuated paraffin and embedded (Fig 7-4 4) The sections are then tested for ferrocyanide as Prussian blue, maximal precautions being taken throughout to prevent diffusion of the highly soluble ferrocyanide. The highly insoluble Prussian blue is observed with the light microscope Ferrocyanide appears exclusively extracellular In the young adult, it is in the form of small droplets about ¼ μ in diameter These occur in groups or clusters which are 1 to 2 μ in diameter (Figs. 7 5*a*,*b*,*c*,*d*)

The smallest units visible with the light microscope (about ¼ μ) were

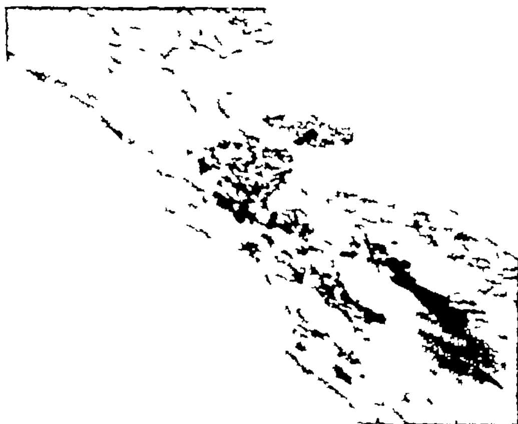


FIG 7-6 Electron micrograph of mouse diaphragm showing ferric ferrocyanide distributed between the muscle fibers, as discrete submicroscopic droplets (×22,000) (*Courtesy of W H Chase A.M.A Arch. Path.*, 67 525–532, 1959)

studied further with the electron microscope As ferric ferrocyanide has a very high molecular weight and seems to be present in high concentration in the droplets, it seemed possible that the ferrocyanide would be sufficiently dense to cast a shadow with the electron microscope To test this possibility the sections studied with the light microscope were re-embedded for study with the electron microscope To do this, slides are passed through xylene (to remove the cover slip and mounting medium) into absolute alcohol and then into 95 per cent alcohol The sections are separated from the slide with a razor blade (Fig 7-4 5) after which they are transferred with a pipette through absolute alcohol (Fig 7-4 6) and into the methacrylate monomer mixture (Fig. 7-4 7) After polymerization (Fig 7-4 8) the methacrylate blocks are sectioned with the ultramicrotome The sections are mounted on grids and observed with the electron microscope. The ferrocyanide droplets visible with the light microscope are now further resolvable into submicroscopic droplets of about 600 to 1200 A (Figs. 7-6, 7 7) [12] which is of the same order as that described by Bondareff

There are several explanations for the localized accumulation in the ground substance of ferrocyanide in the form of droplets The most promising one is based on the fact that the ferrocyanide ion has four negative valences One would expect that this highly charged negative ion would



FIG. 7 7 Electron micrograph of another field showing ferric ferrocyanide between the muscle fibers in the form of submicroscopic droplets. (×36,000.) *(Courtesy of W H Chase A.M.A Arch Path 67 525–532, 1959)*



FIG. 7-8 Photomicrograph of portion of transverse sections of the diaphragm of white Swiss mice showing change in distribution of ferrocyanide which takes place with increasing age of the animal. (*a*) and (*b*) 45 days old. Ferrocyanide is present as droplets which are commonly aggregated to form clusters. The range of normal variation is shown. (*c*) 12 days old. Ferrocyanide occurs largely diffusely and as pools. (*d*) 540 days old. The number of ferrocyanide droplets is much smaller than in younger animals, and they are distributed singly. (×1,200.) (*Courtesy of J. B. Dennis. A.M.A. Arch. Path.*, 67: 533–549, 1959.)

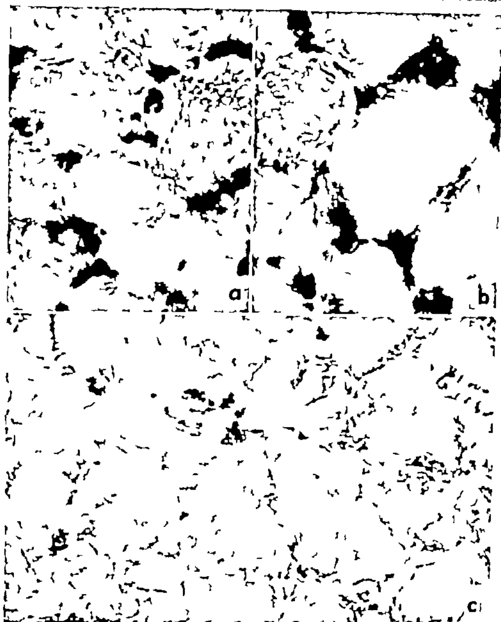


FIG. 7 9 Photomicrograph of portion of transverse section of the diaphragm of white Swiss mice to show change in distribution of ferrocyanide following prior treatment of the animals with hormonal substance. (×1,200) By comparison with the distribution in the form of clusters of droplets in untreated mice (Figs. 7-8*a* and *b*) there is more ferrocyanide in the ground substance of the connective tissue and it appears as large pools and fused droplets after prior treatment of mice with cortisone *a* and with parathyroid extract, *b* Simultaneous treatment of mice with cortisone and vitamin C protects the ground substance from the effects of cortisone and the distribution of ferrocyanide is restricted to discrete droplets as in the normal *c* (*Courtesy of J B Dennis A.M.A Arch Path* 67 533–549 1959)



FIG 7 10 Photomicrographs to illustrate the effect of feeding a rachitogenic diet to rats as compared with an adequate diet, on the distribution of ferrocyanide in the diaphragm. (*a*) Fed rachitogenic diet. (*b*) Fed adequate diet. Feeding of the high calcium, low phosphorus, vitamin D deficient diet for 3 weeks caused the discrete ferrocyanide droplets in the ground substance of the connective tissue to be expanded to form fused droplets and large pools and some to be distributed diffusely (×1,200) (*Courtesy of J B Dennis A.M.A Arch Path* 67 533–549 1959)

tend to be repelled more strongly by the dense phase with its more numerous negative charges than by the water rich phase which has a lower negative charge The accumulation of ferrocyanide in submicroscopic droplets thus seems to be a good indicator of the location and possible amount of water rich phase of the ground substance

The submicroscopic vacuoles containing the electron-dense ferrocyanide may in a sense, be regarded as a positive image the negative image being the less-dense submicroscopic vacuoles of Bondareff When they occur in clusters large enough to form the unit droplets observable with the light microscope the unit droplets (about $\frac{1}{4}$ μ) may then be used as an indicator of the relative composition of water rich and colloid-rich phases of the ground substance of the connective tissue As studies with the light microscope are relatively free of sampling errors which commonly arise in studies with the electron microscope, the way seemed clear to an analysis of the factors which influence the relative proportions of water-rich and colloid rich phases in the ground substance.

Dennis has studied some of these factors [13] His most pertinent results may be summarized as follows

1 The distribution of ferrocyanide shows a progressive change with age (Figs. 7-8*a*,*b*,*c*,*d*) In the diaphragm of white Swiss mice 4 to 12 days old, ferrocyanide is distributed in the ground substance in part diffusely

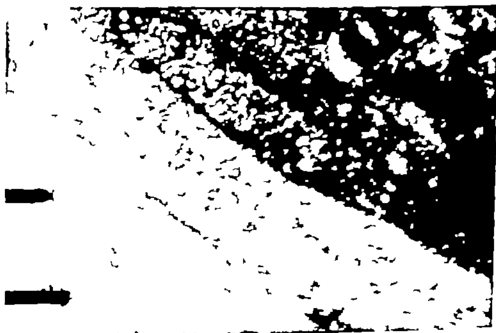


FIG 7 11 Electron micrograph of a portion of a cartilaginous spicule to show the vacuolar nature of the osteoid deposited between an osteoblast (upper right) and a cartilage remnant (lower left) The osteoid consists of regions of low density enclosed in denser walls. ($\times$20 000) (*Courtesy of W C Durning J Ultrastruct Res.*, 2, 245–260 1958)

and in part as pools. At 24 days, some enlarged droplets are present in addition At 30 days, the diffuse component is absent At 45 days and later visible ferrocyanide is confined entirely to the unit droplets occurring in clusters as described by Chase At 540 days the ferrocyanide droplets are greatly reduced in number and occur largely or entirely as unit droplets. It would seem then that the water rich phase of ground substance of connective tissue decreases progressively during maturation and aging in this animal

2 Following treatment of young adult mice with estrogen, DOCA cortisone, and parathyroid extract (Figs 7 9*a*,*b*) visible ferrocyanide is more diffusely distributed than in untreated controls, and the unit droplets are not to be seen The same result takes place in more exaggerated form in rachitic rats reared on a diet high in calcium, low in phosphorus, and deficient in vitamin D (Fig 7 10*a*,*b*) It would seem, then that in all these substances the water rich phase of the ground substance is increased The generalized effect of parathyroid extract was not altogether unexpected [4] More surprising was the apparently generalized effect on the ground substance of the rachitogenic diet

3 Vitamin C has no apparent effect on the distribution of ferrocyanide in the ground substance of the diaphragm of otherwise untreated mice However when given simultaneously with cortisone vitamin C suppresses



FIG. 7 12. Electron micrograph of a portion of a bony spicule to show the less dense and more dense regions of the osteoid. Portion of an osteoblast is above bone is below the osteoid. Fibrils with the periodicity of collagen are present in the denser phase of the osteoid. (×24 000) (*Courtesy of H C Durning J Ultrastruct Res.*, 2, 245–260 1958)

the effect of the hormone (Fig 7 9c) Whether this effect is specific, or takes place also with other agents which increase the water rich phase of the ground substance is not known

Before embarking on some observations on osteoid, I should like to summarize briefly the major findings on the nature of the ground substance

1 Ground substance is organized as a system of discontinuous water rich colloid-poor submicroscopic vacuoles These are separated by the denser water poor colloid-rich continuum These two elements of the ground substance are considered to be the morphologic expression of a two-phase system The relative proportions of water rich and colloid-rich components may be readily altered, as during maturation and aging after the administration of various hormonal substances and, under certain conditions, by vitamin C As the two phases are assumed to be in thermodynamic equilibrium changes in the relative proportions of each phase will result in changes in the concentration of the ions of the ground substance.

2 The colloid-rich phase seems to be the site in the ground substance where collagen fibrils originate

The more general aspects have been extended in studies on osteoid

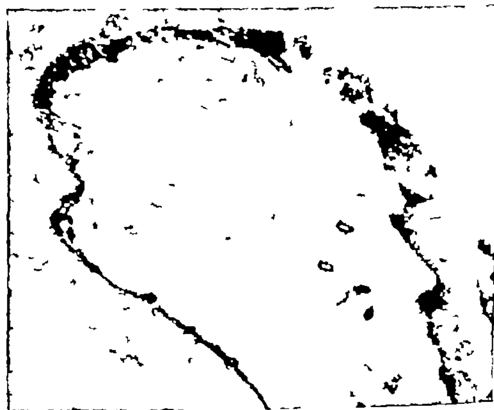


FIG. 7 13 Electron micrograph of a portion of a bony spicule to show the presence of ferrocyanide in the osteoid border (×7 000) (*Courtesy of W H Chase*)

of developing bone Durning[14] has shown that the osteoid border of normal bone spicules of the rat contains the elements of a water rich and colloid rich two-phase system resembling closely that of the ground substance of rat tail tendon (Figs. 7 11 7 12) His studies also suggest that collagen fibrils arise in the denser phase again as in rat tail tendon (Fig 7 12) Chase[15] has shown that ferrocyanide accumulates in this same

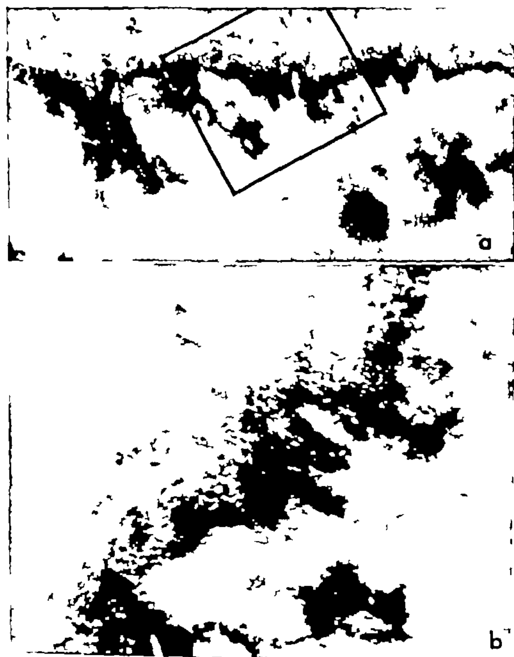


FIG 7 14 (a) Electron micrograph of a portion of a bony spicule to show the presence of ferrocyanide occurring as dense deposits in particulate form (×7 000) (b) Enlargement of portion enclosed in a (×36 000) (*Courtesy of W H Chase*)

osteoid border (Figs. 7 13 7 14) On the basis of the foregoing, it is clear that normal osteoid could be regarded as a form of ground substance of connective tissue It seems probable that the findings on the factors influencing the physical and *chemical lability of the ground substance of muscle,* which are more readily subject to study may be extended to osteoid.

The point of view which I have expressed emphasizes the possibility that a fuller knowledge of the ground substance of connective tissue can be expected to contribute toward an understanding of the dynamics of calcification. Out of this ground substance, which is osteoid almost simultaneously arise the collagen fibrils and the smallest bone salts. Yet it is true that a two-phase system and collagen fibrils are present in both tendon and bone The crucial question then becomes Why does tendon generally not calcify normally while bone does calcify? A large part of the answer I believe will be found in the dynamics and transformations of ground substance

The correct answer to the question is not easy to get at. It could lie in the different chemical composition or physical properties of the collagen or the ground substance in tendon and bone It is also possible that if there are differences they have no effect on calcifiability and that other factors are involved. One such factor is the element of rate Molnar [16] especially is studying the possibility that the rate of formation and maturation of ground substance is the deciding factor in determining whether a tissue becomes calcified The working hypothesis is that ground substance matures more slowly in tendon than in bone The evidence for this is rather weak, *and an effort is being made to ascertain whether such a difference occurs.* The term *maturation of ground substance* is used to describe the transformation of a water rich ground substance to a water poor state that is, the transformation of a ground substance with a relatively high proportion of submicroscopic water rich droplets to a state with a markedly lower proportion of these droplets

Let us, however suppose that the rate of maturation of ground substance is appreciably greater in bone than in tendon As the water rich phase is transformed to a colloid-dense phase the total calcium content locally would tend to be increased by a factor of 3 If now one considers that collagen fibrils are being formed from tropocollagen in the dense phase one could assume that collagen would take with it a certain fraction of water and that this water would not be available for solution of the calcium-colloid complex In effect, this amounts to a concentration of the calcium-containing fluids The amount of this concentration is not known, but if it were large enough, the calcium compounds would tend to become *insoluble* as *from a supersaturated solution* and one would expect to find that the first calcium deposit should be amorphous Dr Molnar finds that this does in fact seem to be the case calcium appearing in noncrystalline form in and around the freshly formed collagen fibrils. The

same process would not happen in tendon according to this hypothesis because the whole process is slower and equilibration with the tissue fluid could take place more effectively thus resulting in a loss of excess calcium from the affected region It is difficult to say just how much this concentrating effect amounts to depending on the assumptions made the concentrating factor could vary from 2 to 20 times This, combined with the density change, would yield a concentration of total calcium in osteoid of 5 or 6 to 50 or 60 times According to Neuman and Neuman,[16] a local concentration of calcium greater than three times could influence nucleation. The method described would supplement the nucleation phenomena described by Neuman and Neuman and by Glimcher et al [17] A significant aspect of the proposed hypothesis is that collagen fiber formation and the deposition of a reservoir of insoluble calcium take place simultaneously with the latter incorporated within and deposited on the newly forming fibrils.

Acknowledgments

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8

Microscopic Metabolism of Calcium in Bone*

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The various processes involved in the mineral metabolism of the skele ton have been recently reviewed by Neuman and Neuman.[1] They have shown that new crystal formation recrystallization, crystal surface ex change intracrystalline exchange crystal growth resorption, and the aging of each element of bone structure probably all contribute to the complexity of interrelated events occurring in the skeleton. The problem before us is to isolate some of these processes for quantitative study in living animals

The first problem of isolation is one of location We would like to concentrate our attention on individual regions of bone that are as homogeneous as possible with respect to their structure to their stage of development, and to the metabolic processes taking place within them By focusing our attention on microscopic volumes of bone we can hope to obtain more definitive results than those obtained by measurement of the average properties of large volumes of bone Our method is to inject an animal intra venously with Ca^{45} sacrifice it after an appropriate interval, and then analyze the bone by quantitative autoradiography and quantitative x-ray microradiography These techniques permit us to study the calcium me tabolism of volumes of bone 10 to 100 μ on a side We are therefore able to avoid resorption cavities or Haversian systems of unknown age and thus eliminate from our analysis for Ca^{45} the effects of resorption.

From such microscopic measurements we can only indirectly identify processes at the molecular level, so that it is important to use concepts and terminology in describing our data that do not go beyond the resolu tion of our measurements. First, we will define a *microscopic volume* of bone as a cube 10 to 100 μ on a side in which no resorption is taking place We will use the term *accretion* to describe any process involving

* Work performed under the auspices of the United States Atomic Energy Commission.

the net transfer of calcium to a single microscopic volume of bone We will use the term *exchange* to describe any process involving equal and opposite rates of transfer of calcium atoms to and from a single microscopic volume of bone These definitions of accretion and exchange can be employed unambiguously to whole bones or to the skeleton, because one then considers merely the sum of many microscopic volumes Exchange under this definition includes the molecular processes of ion exchange intracrystalline diffusion and recrystallization Note that this definition of exchange does not specify how long it may take for Ca^{45} atoms to reach equilibrium following their injection on one side of the exchange process. In our present terminology we refer to short-term or long-term exchange, depending on whether the time necessary for the attainment of radioactive equilibrium is much shorter or much longer than a week

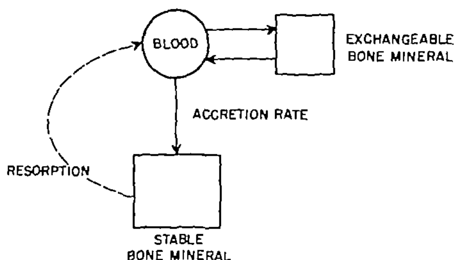


FIG 8-1 Bauer Carlsson, Lindquist model for calcium transfer between blood and bone.

Now we need a way to isolate the processes of exchange and of accretion in a given microscopic volume of bone from all the other processes taking place in different parts of the animal we need to eliminate from our calculation the effect of deposition, resorption, or exchange of Ca^{45} elsewhere in the skeleton and the effect of Ca^{45} excretion from the body as a whole This can be done by using in our calculations measured values of the specific activity of the blood If we know the ratio of the number of Ca^{45} atoms to the number of Ca^{40} atoms in the blood throughout a given experiment then no matter how the Ca^{45} may have been introduced into the system (whether by multiple or single injections or by continuous feeding) and no matter what processes of uptake or elimination of Ca^{45} may be going on in other locations, we can calculate from the quantity of Ca^{45} which we find in our microscopic volume of bone the rate of calcium

transfer which has existed between the blood and this volume of bone during the experiment

Bauer Carlsson and Lindquist[3] have developed a fruitful technique of analysis which derives its power from this use of observed blood specific activities Figure 8 1 shows the physical model underlying this analysis. Bone mineral is divided into two fractions stable bone and exchangeable bone Their exchangeable fraction refers to short term exchange, that is,

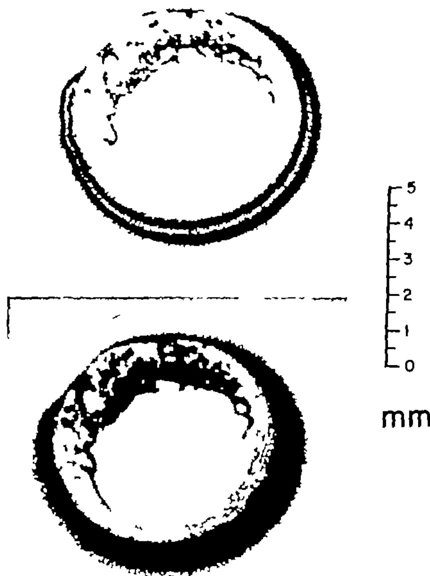


FIG. 8 2 Autoradiograph of a cross section through the midshaft of the femur of a puppy injected with Ca^{45} intravenously at age 2 months, again at age 3 months, and sacrificed at age 4 months. *Upper* short exposure *Lower* longer exposure.

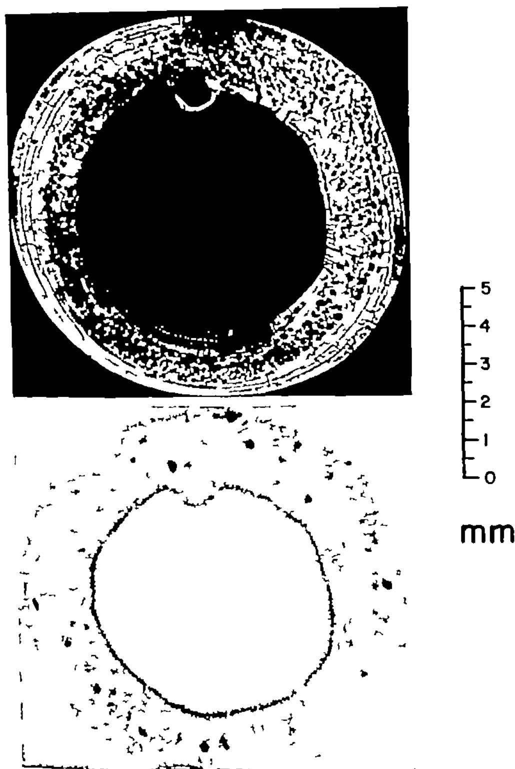


FIG. 8-3 Corresponding autoradiograph (*lower*) and microradiograph (*upper*) of a cross section through the midshaft of the femur of an adult dog injected with Ca^{45} and sacrificed 2 weeks after injection. Similar autoradiographs have been obtained up to a year after injection (*By permission of J H Marshall R. E Rowland and J Jowsey Radiation Research* 10 213–270 1959)

to exchange which has a short time constant and consequently reaches radioactive equilibrium with blood within minutes or a few hours after injection. The Bauer Carlsson, Lindquist analysis provides a way to separate the processes of short-term exchange from any other processes of calcium transfer When used together with quantitative autoradiography the analysis is particularly simplified since by choice of location we can avoid the influence of resorption upon a measured Ca^{45} activity Thus we can wait weeks after a Ca^{45} injection before sacrificing an animal for analysis, and by this time the specific activity of the blood has dropped to such low values that there is a negligible amount of Ca^{45} in the short-term exchangeable fraction of this bone The rate of Ca transfer from blood to stable bone is then obtained by dividing the observed Ca^{45} content of the bone by the time integral of the specific activity of the blood during the experiment. Bauer has shown that in growing bone this rate of calcium transfer corresponds closely to the accretion rate As Neuman has pointed out, and as I will show this correspondence does not always hold but first let me describe a microscopic experiment on growing bone

Figure 8-2 shows a Ca^{45} autoradiograph of a cross section through the mid-shaft of the femur of a young puppy This puppy was injected with Ca^{45} intravenously at the age of 2 months, again at the age of 3 months, and was sacrificed at the age of 4 months The upper autoradiograph has been exposed to show the rings of Ca^{45} activity taken up during periosteal growth at 2 months and 3 months of age The lower autoradiograph has been overexposed to show the diffuse uptake of Ca^{45} in bone formed before the first injection. Since the animal was sacrificed a month after the second injection we can use the quantity of Ca^{45} in either of these two rings to calculate the accretion rate according to the Bauer Carlsson Lindquist method The intensity of the first or inner ring yields an accretion rate equivalent to 16 μ/day the second or outer ring a rate of 10 μ/day The average rate during the month between injections was thus 13 μ/day and this rate is confirmed by measurement of the distance between the two rings, at least within the 20 per cent accuracy of the measurements. Thus at this growing periosteal surface of bone the accretion of calcium predominates over the processes of long-term exchange discussed by Neuman. However this is not the case in the bone that had already been formed before the first injection

The diffuse uptake of Ca^{45} in bone existing before injection is better demonstrated in an adult dog Figure 8 3 shows at the bottom an autoradiograph of a cross section through the mid-shaft of the femur of a dog a few years of age which was given a single intravenous injection of Ca^{45} and sacrificed 2 weeks after injection The upper part of this figure shows the corresponding x ray microradiograph. With an adult animal such as this we may choose a microscopic volume of bone in which no new bone formation is taking place and yet there is still a Ca^{45} uptake by stable

bone (Remember that the short term exchangeable fraction of this bone will contain negligible Ca^{45} 2 weeks after injection) According to the Bauer Carlsson Lindquist analysis the accretion rate in this bone is 0 2 Gm Ca per gram Ca per year In other words, the Ca^{45} uptake indicates that the mineral density of this bone should be increasing some 20 per cent per year Now you will notice on the microradiograph that some of the re gions of this bone contain secondary Haversian systems, and from the beautiful work of Amprino[7] we know that over long periods of time Haver sian systems do increase in mineral density However Fig 8 3 shows broad areas of cortical bone that were laid down periosteally and have not been re placed by secondary Haversian systems. Such bone is often referred to as fully mineralized bone But we can see that its diffuse uptake of Ca^{45} is only slightly less than that in the regions of secondary Haversian bone Could it be that this periosteal bone is also slowly increasing in calcium density at a rate comparable to the measured rate of calcium transfer?

To answer this question we have made microscopic measurements on corresponding volumes of canine periosteal bone of various ages Figure 8-4 shows the results of the autoradiographic measurements The ordinate is the rate of long-term Ca transfer from blood to bone calculated from

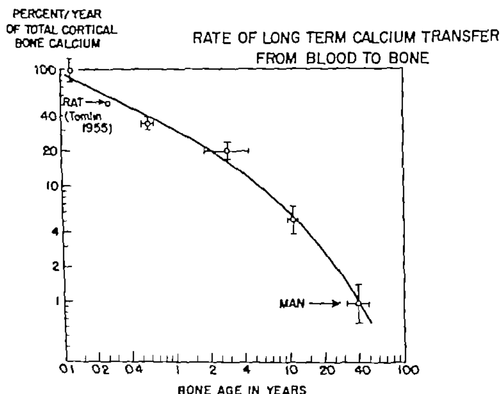


FIG 8-4 Rate of long term calcium transfer from blood to bone for microscopic volumes of canine periosteal bone measured by means of Ca^{45} diffuse uptake A single measurement for a man dying of radium poisoning is tentatively in cluded.

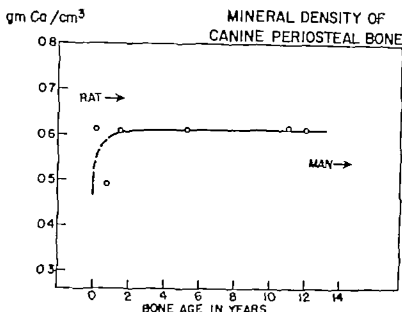


FIG. 8-5 Microradiographic measurements of the calcium content of microscopic volumes of canine periosteal bone. Preliminary measurements for rat and man indicate a shift in the curve to the levels indicated.

the observed Ca^{45} content weeks after injection According to the Bauer Carlsson, Lindquist model, this rate of transfer should be identical with the accretion rate The abscissa is the age of this bone, a few months less than the age of the dog It is interesting that the rate of Ca transfer from blood to the cortical bone of 3-month-old rats measured by Tomlin Henry and Kon[3] using continual Ca^{45} feeding is consistent with the present data.* A single measurement from the Ca^{45} diffuse uptake in cortical bone of a 50-year-old man is tentatively included on this same graph Now the most significant thing about Fig 8-4 is that the rate of Ca transfer is much too large to represent pure accretion For example microscopic volumes of periosteal bone 6 years of age would have to contain 50 per cent more calcium than periosteal bone 2 years of age We have directly measured the calcium content of this periosteal bone as a function of bone age by means of quantitative microradiography The results are shown in Fig. 8-5 It is clear that, at more than a few months after its formation this bone shows *an almost constant microscopic calcium density* This remarkable paradox has been pointed out by Arnold, Jee and Johnson,[4] but here we have it in quantitative form

From the diffuse uptake of Ca^{45} we have measured a rate of calcium transfer from blood to microscopic volumes of bone in which no resorption is occurring and yet there is no corresponding increase in their calcium content We are forced to conclude that there exists a comparable rate of

* These authors interpret the observed Ca transfer to be predominantly accretion but microradiographic measurements were not made.

calcium transfer back to blood and that most of the observed Ca^{45} is therefore engaged in an exchange process But if this is so how could this Ca^{45} be retained in bone for weeks or months after Ca^{45} injection? This must be an exchange in which each exchanging calcium atom spends on the average more than a year in bone before returning to the blood In other words, for long periods of time after injection radioactive equilibrium is not attained We are therefore observing a process of *long-term exchange*

Under what conditions is it possible to explain this long retention of Ca^{45} in an exchange process? The currently accepted explanation is essentially that the rate of exchange decreases so rapidly with bone age that Ca^{45} is trapped on the bone side of the exchange The rate of exchange certainly does decrease but this is not a sufficient explanation for bone a few years of age the decrease in rate during a single year is not large (Fig 8-4) and so cannot be primarily responsible for the long Ca^{45} retention. We are left with the conclusion that the exchange itself as a long time constant, or in other words, that the exchangeable fraction—the calcium available for this exchange—is a large fraction of all the calcium in any microscopic volume of mature bone

It is not obvious how such a process of exchange could take up Ca^{45} within a day after injection and then hold it for a year or more To understand this we must consider the actual magnitudes of the blood and bone specific activities Because some of the following experiments have employed Ra^{226} instead of Ca^{45} as the tracer I will refer to either Ca^{45} or radium as calcium like radioisotopes Their blood specific activity curves[8] and their diffuse uptake in dogs we find to be quite similar Figure 8-6 shows a typical curve for blood specific activity in a 1 year-old dog fol

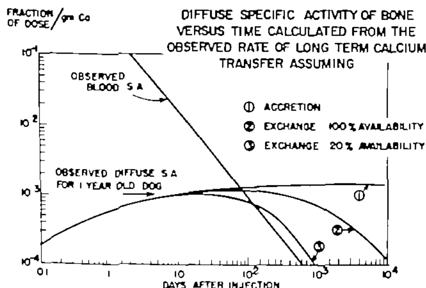


FIG. 8-6 Calculated values for the Ca^{45} or Ra^{226} diffuse specific activity of canine periosteal bone 1 year old as described in text.

lowing a single injection of a calcium-like radioisotope The ordinate is th fraction of the total activity injected per gram of calcium in the blood. The diffuse specific activity in the cortical bone of a 1 year-old dog is little less than 10^{-3} of the dose per gram calcium so that during the fir day after injection the specific activity of the blood exceeds that of a micr scopic volume of nongrowing bone by a factor of 1 000 Therefore th transfer of Ca^{45} from blood to bone can proceed rapidly despite the lon term nature of the exchange

Now how long could Ca^{45} be expected to be retained? We know th rate of exchange as a function of bone age (Fig 8-4),† and we kno the specific activity of the blood as a function of time after injection. There fore, we can calculate the time dependence of the specific activity of microscopic volume of bone

CALCULATION FOR FIGURE 8-6

The following differential equation, representing one bone compartmen coupled to blood by a decreasing rate of calcium transfer was solved fo the initial condition $v = 0$ at $t = 0$

$$\frac{dv}{dt} = f\left(B - \frac{v}{\alpha}\right)$$

where v = average specific activity of a microscopic volume of bone as a function of time

B = observed specific activity of the blood as a function of time

f = observed rate of long-term calcium transfer as a function o time (taken from Fig 8-4)

α = fraction of the calcium content of the microscopic volume taking part in the exchange

t = time measured from time of injection

Bone age at time of injection taken as 1 year
Curve 1 (accretion) calculated setting $\alpha = \infty$ (or $v/\alpha = 0$)
Curve 2 $\alpha = 1$
Curve 3 $\alpha = 0\,2$

For simplicity we assume that bone can be represented by a single compartment model.‡ Clearly the longest retention would be produced if all the calcium in the microscopic volume were taking part in the exchange this case is shown by curve 2, Fig 8-6, for a dog injected at 1 year of age.

A 9-kg dog has a blood content of about 0 05 Gm Ca and a skeletal content of about 140 Gm Ca.

† To be precise, we know that for canine periosteal bone at least the middle part of the curve in Fig. 8-4 (1 to 10 years) represents primarily long-term exchange.

‡ More complex models are treated in Argonne National Laboratory Radiological Physics Division Semiannual Report No. ANL 5919 (in press) but they show that the essential point is the large compartment behavior of the exchange

If only 20 per cent of the calcium were involved then the specific activity of the microscopic volume would decrease more quickly as in curve 3, Fig. 8-6 For comparison curve 1 Fig 8-6 shows the behavior to be expected if the uptake of Ca^{45} were due primarily to accretion and not to long-term exchange, though this possibility has been ruled out by the x ray measurements.

Now notice three things about this calculation (1) With experiments of much less than a year s duration it is impossible to tell from the behavior of the radioactive tracer whether it has been deposited in bone by a process of accretion or is only temporarily deposited in a process of long-term exchange (2) Since we know that diffuse activity appears fixed in bone for many months after injection we are forced to conclude that the exchangeable fraction for this long-term exchange must be a large fraction of the total cortical bone calcium (3) No matter how large an exchangeable fraction we assume, this process of exchange requires that over periods of years an appreciable loss of calcium like radioactivity should occur even from mature bone without the influence of resorption

This prediction has now been confirmed in three experiments (1) A 1 year-old beagle was injected with Ca^{45} and his left radius-ulna amputated at 1 month as a control At 1 year after injection the radius of the opposite leg showed a 10 to 20 per cent loss of diffuse activity in regions not subject to resorption (2) A group of 11 beagles were injected with radium and autoradiographically analyzed for diffuse activity at different times after injection The animals sacrificed 4 years after injection show approximately one half the diffuse specific activity of the animals sacrificed a few months after injection. (3) Bone from radium injected human beings has been examined 25 to 30 years after injection and shows that Haversian systems laid down at the time of injection have lost by exchange about 90 per cent of their original radium content.[6] These experiments are being reported in detail elsewhere [8]

As to the mechanism for this long-term exchange it probably involves an exchange at crystallite surfaces, possibly coupled with intracrystalline diffusion, and perhaps accompanied by recrystallization Detailed analysis of long-term tracer experiments may be able to distinguish between these possibilities. The physical explanation for the existence of a large calcium compartment in exchange with blood is provided by the extremely small size of bone crystallites in which a large fraction of the calcium is in or near the crystallite surfaces.

Summary and Conclusions

To sum up we have shown that the uptake and retention of calcium like radioisotopes by mature cortical bone in dogs *cannot* be explained as a process of accretion and therefore must be predominantly a process of

long-term exchange The measured rates of this exchange as a function of bone age require that over a period of years the radioactivity should begin exchanging back again to the blood, and this prediction has now been confirmed by three experiments. Although I have discussed this long-term exchange primarily with regard to the diffuse uptake in mature bone it should also produce a long term loss of radioactivity from bone formed during the injection period, though the time dependence may not be the same

The existence of long-term exchange has three effects upon our concepts of skeletal metabolism First, as shown in Fig 8 7 the Bauer, Carlsson,

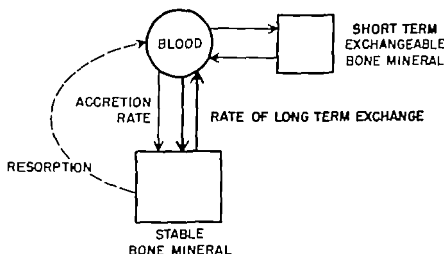


FIG 8 7 Suggested model for calcium transfer between blood and bone.

Lindquist analysis includes in its measurement of accretion rate the transfer rate of long-term exchange This may not be a serious correction to skeletal accretion rate measurements in practice, because whenever bone growth is occurring the accretion rate for calcium should outweigh the rate of long-term calcium exchange Second, mature bone is not completely diffusion-locked and inert but is able to maintain a slow exchange of Ca with the blood Since there is much more mature bone than young bone in an adult skeleton the contribution of the large mass of mature bone to the blood bone interchange of Ca is not negligible In adult dogs, long-term exchange with mature bone turns over two to three blood volumes of Ca per day And finally calcium like radioactive isotopes deposited in the skeleton will over periods of years diffuse out of even mature bone because of long-term exchange

Acknowledgments

I would like to thank the following collaborators in this research Robert Rowland, Jenifer Jowsey Webster Jee Robert Flynn, and Robert Hasterlik. I am deeply indebted for continued advice and discussion to L. D Marinelli and Franklin D McLean.

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DISCUSSION

Dynamics of Calcium Metabolism

Chairman Cyril L. Comar Ph.D

CHAIRMAN COMAR Before we proceed with the discussion, I would like to make one or two comments In Geneva after Dr Bauer gave a similar talk, a young worker from Israel got up and described a technique which I think might be very useful and important. He had been going back to the use of alizarin to demonstrate calcification and had realized that if one could label alizarin with radioactive iodine then one might be able to do the same thing with alizarin as with Ca^{45} Sr^{85} or Ca^{47} As I understood it, his results so far had agreed very well with those of Dr Bauer Ironically this seems to complete the cycle—alizarin yellow to radioisotopes and back to alizarin

Also I would like to take this opportunity of developing the background of some of the work that Dr Bauer and Dr Neuman have also described. In the beginning the grand old man of radiochemistry George Hevesy looking at phosphorus deposition in bone sensed that one couldn t account for this entirely by growth and felt that exchange must be involved Of course Dr Hevesy didn t know much about rates of accretion and rates of resorption, but he intuitively felt that exchange must be a major factor

This concept was developed in major part by the University of Rochester group Harold Hodge and Bill Neuman extended, developed and applied these concepts vigorously

A few years later the Swedish group Carlsson Bauer and Lindquist came up with the idea that one should look at growth as being primarily

responsible for these materials getting into the skeleton, and so their lit thought developed along these lines. There has been some controversy the past 3 or 4 years I don t think Neuman and Bauer will come to b this afternoon because the thinking is getting a little closer and each gi is realizing, I think, that both mechanisms are probably operative. Th think, is a very fine thing It demonstrates that one should not take granted any authoritative word that is set forth, regardless of who authority is We should never allow ourselves, in our thinking and in research, to be restricted by ideas of others, but should go ahead and velop as personal inclination dictates.

Now we are ready for the discussion. I think we will start off Dr Neuman if he will read the question and give the right answer

DR. NEUMAN "Has the fraction of serum cleared of calcium by kidney been calculated yet?"

To my knowledge there have not been many studies of clearance calcium by the kidney The reabsorption of calcium in dog, at least normal dog, is 99 95 per cent—a fantastic return fantastically effic reabsorption Therefore the clearance is essentially negligible in the nor dog and in the normal human being

I have another question "Did the 640 banded collagen which calcify contain mucopolysaccharide?"

I am not an expert on this but, to my knowledge, no one has ever p fied collagen to the extent that it did not contain some hexosamines, if that means that mucopolysaccharide is present, no one ever obtai collagen free of mucopolysaccharide.

"Can highly purified 640 banded collagen be prepared?"

As a matter of definition once you have purified it, it is pure and would say yes.

"Will it calcify under appropriate conditions?"

The answer is yes. I believe I quote Glimcher correctly in that the m purified the collagen, the better it calcifies, and ours recrystallized sev times does better than less purified material.

And finally a question by Dr Bachra "What is the evidence for inhibitory action of mucopolysaccharides on the calcification of collage

I am not sure whether he is kidding me because I recall I saw a slide a Gordon Conference which indicated that in Dr Bachra's data. If recollection is correct, I would turn to the evidence of Glimcher fro again, an over-the-table discussion Certainly the collagen prepared direc from connective tissue which contains a lot of mucopolysaccharide d not seed and after purification it does

DR. BAUER I have some sixteen questions to answer but some of th pertain to the same thing Dr Urist asks, "Is osteoporosis due mainly resorption?"

If Dr Urist means by osteoporosis the same thing that Dr Nordin mea

by osteoporosis, which is not the same thing that Dr Reifenstein means by osteoporosis Dr Urist uses this term to mean less bone than normal Obviously you cannot have less bone than normal unless resorption has been going on somewhat faster than formation

Dr Katz asks, "What is your operational definition of exchangeable and nonexchangeable calcium?"

It has been found useful to introduce two concepts, exchangeable and nonexchangeable calcium because if one measures the amount of radioactive calcium in bone following injection of radioactive calcium to animals or to man, it has been found that the activity in bone is the sum of two functions. One is *a function of the specific* activity of blood radioactive calcium, and the other is a function of integrated specific activity of blood radioactive calcium.

Dr Spencer asks a technical question "How thick is the lead shield needed to exclude penetration of gamma rays originating from a distant area when a localized area is surveyed?"

Answer It should be 5 to 8 cm thick.

"Can you quantitatively estimate the amount of radioactive calcium or Ca^{47} or Sr^{85} collected at an individual point in bone?"

It is possible to measure the absolute amount of radioactivity in, for instance, the leg or part of a leg of a man in vivo and it is possible of course to dissect out a piece of bone in a rat, but at a specific point in bone it is not possible to measure the absolute activity by the external counting technique

CHAIRMAN COMAR May I comment, Dr Bauer? There are some collimating scintillation detectors available that I think will eventually enable one to measure only the radiation from a given volume in space Of course, you usually have to use more activity in a situation like this but theoretically it may be possible

DR BAUER You mean the so-called focusing mechanism?

CHAIRMAN COMAR Yes, the flat field detectors.

DR BAUER Question "Have you determined *in experimental animals* the ratio of accumulation of radioactive salts in the fractured bone as contrasted with the traumatized tissue immediately surrounding the fracture?"

This problem is referred to also in a question by Dr Stevens "Do you take into account in your external counting that there may be differences in total blood flow as between diseased and contralateral normal (control) bone?"

And two questions by Dr Spencer (1) "How do you separate the radioactivity in blood flowing through muscle when *you count externally* muscle and bone?" (2) "May the high radioactivity reported in the Paget lesion be due at least in part, to the arteriovenous fistulas in bone so frequently present in Paget's disease?"

[Slide] This is from a woman with a Paget lesion in her left tibia and it

for ferrocyanide It is different from that of chloride in muscle This is more uniformly distributed in the ground substance

"Bélanger found stronger Ca^{45} in immature cartilage of epiphyseal plates of rachitic chicks than in sites where healing can occur How does this fit into your hypothesis?"

I don t think the explanation for calcification of cartilage is the same as that proposed for calcification of osteoid

This is from Dr Dorset "What do you postulate is the role of the osteoblast in the process of osteoid maturation and calcification?"

As I visualize it, the osteoblast is an awfully busy cell It is busy making stuff and secreting it and then it is busy influencing it though the nature of the influence is unknown

"Would it not be equally justifiable to conclude that the groups of ferrocyanide granules represent simply random loose precipitates rather than an affinity for water rich or water poor colloidal regions?"

That, I think, is very unlikely It s like the first question

"Could you not verify your hypothesis by performing test tube experiments with suitably prepared colloids, water rich and water poor?"

I suppose it is possible to set up such a system

CHAIRMAN COMAR Before we go to the next panelist, Dr Bronner would like to comment briefly on the term *exchange*

DR. FELIX BRONNER Thank you It is just because I have been so thoroughly confused by the various types of exchange that we have been talking about in this field that I would like to make a plea to Dr Marshall to use a different term than exchange If I understood his very excellent presentation correctly you have a large pool of calcium essentially in bone and there enters into it calcium that we have labeled or that previously has been labeled and it is essentially a statistical process A certain fraction of what enters this pool is compensated by a fraction that leaves it. The net amount is what we then call accretion.

I personally feel it would be better not to call this general process exchange because to me an exchange is an ion for ion exchange You can have a heteroionic or homoionic exchange but you would expect that the very same time that an ion takes a place (and I think this is the way that Bill Neuman used the term originally) as an ion enters from blood or from one system to the other system at the very same time another ion leaves to make room for this place to make room for the entering ion.

I feel that if we restrict the term "exchange" to this latter process we should find some other term to describe what you have called the long term exchange of diffusion I am in thorough agreement with your interpretation of your findings but I did want to make a plea for a different term

DR. MARSHALL I think this a far from trivial point and I don't want to insist on this way of describing the process However there are two rea

sons for using the term First, in addition to its special meaning giv Dr Bronner exchange is in general use as an operational concept. I broader sense exchange is defined as a transfer of atoms or ions in directions without a net transfer The second reason is that the conc exchange has led to some disagreement which the present observ may help to clarify

The point is that when you are talking about exchange you ha refer to your scale of observation, to the volume within which no net t fer occurs. Dr Bronner refers to a single ionic site I refer to the sm volume resolved by the tracer technique This concept of exchange t from a microscopic volume is valuable because it allows you to describ results of tracer measurements without confining yourself to a parti explanatory mechanism such as diffusion or recrystallization

In addition, to describe an exchange or an exchangeable fra uniquely you need a time factor the mean time of residence of an ato the bone side of the exchange Both Dr Neuman and Drs. Bauer Carl and Lindquist have made important contributions to the measureme exchange in bone mineral and both have used operational definitio exchange I believe the essential difference is the time factor Dr B refers to short term exchange I refer to long-term exchange, and Neuman refers to both ("Short" and "long" refer to less than and i than a week.)

Dr Arvid Carlsson asks, "How do you measure the activity in one the same osteon at two different intervals?"

This, I wish I knew because this is what we would like to do next can possibly do it by giving one tracer measurement, waiting giving other tracer measurement and then looking at the same osteon, but isn't really the question I have to plead that I don't know of any way

In the work that I have described the only way that we have been to measure the age of this bone was by avoiding osteons and lookin bone that was laid down periosteally

DR. BAUER I happen to know why Dr Carlsson asked that ques The reason was that John Marshall said that, in a man who died som years after he had received radium he found that in one osteon abou per cent of the activity that was originally present in that osteon had di peared from the osteon. This would mean that Dr Marshall had meas this osteon twice so I guess that is the reason why Dr Carlsson as the question.

DR. MARSHALL Yes, that of course was not two direct measurement full description of that measurement appears in the proceedings of the 1 Geneva Conference The observation applies to hundreds of osteon three different subjects. The essential point is that the human bei formed the series of Elgin cases that were given weekly injections of rad for many months Osteons that form during the injection period buil

radium at the specific activity of the blood or close to it This has been checked by experiments on dogs.

Since we know the amount of radium per injection we can calculate at least roughly what specific activity those osteons should have and the level we find now in practically all the osteons containing significant amounts of radium is one tenth of that predicted level If these were osteons that had formed before injection there should have been at least a few growing at the time of injection, but as far as we search in the bone we find no osteons with more than one-tenth the predicted level If these osteons formed more than several weeks after the last injection they could not have even one tenth the predicted activity

Turning to the question given Dr Bauer by Dr Kengy "Can the isotopes be traced to the immediate area of osteoblasts by any type of microscopic work?"

Yes. Autoradiography of such resolution is certainly possible The autoradiographs which I showed are not representative of high resolution work because quantitative measurements are more easily made by low resolu tion techniques

But I am sure Dr Lacroix is going to show us autoradiographs tomorrow with much higher resolution and Dr James Arnold recently has shown plutonium concentrations in separate cells, although these were osteoclasts

This is a question by Felix Bronner "Essentially what do we know of bone that is very young decreasing the age of the animal below about an age of 1 year?"

We know that the rate of long term exchange increases with decreasing age but for very young bone we have not found how to make good measure ments. The uptake of activity by accretion in nearby growing bone domi nates.

CHAIRMAN COMAR Dr Nordin, did you want to comment?"

DR. B E C. NORDIN On Dr Neuman's point about the calcium clearance in dogs, I didn't want to let that go unchallenged Dr Sabin and I have been talking about it and of course if the human should reabsorb 99 9 per cent of calcium there would be no calcium in the urine and osteoporosis would not develop The whole point is that the calcium clearance must certainly be different from that in man, and it is something of the order of mils per minute or approximately 2 per cent of the calcium that is not replaced 98 per cent is reabsorbed 2 per cent is not.

Now the figure is really compatible with ordinary observations in man

DR NEUMAN You caught me without a pencil so I can't argue I believe you have a pencil or at least have had time to do a mental calculation

I have a question from Dr Copp which is a mean one for a friend to give "What is the percentage of calcium in the hydration shell?"

Of course there is no fixed percentage of calcium in the hydration shell. It is dependent on the solution conditions under which the measurement

is made but as a rough order of magnitude the concentration of calcium in the hydration shell under ordinary conditions of ion strength is nearly one thousand times that of the bulk solution around it, and just what the state of this calcium is is a little difficult to say at the present time It is a rather confusing one

I can't help but take one moment to comment We have been very polite here. The question of terminology is one that I view with some amusement because I have suffered with the term recrystallization" for at least 6 or 8 years, and I am going to watch with more than ordinary compassion the life span of the term "long-term exchange"

The other aspect was something that we haven t had in the open too much the long and friendly argument concerning the measurements of accretion rates by in vivo measurements I have maintained that this is lifting oneself by one s bootstraps and Dr Bauer and Dr Carlsson, I think have successfully proved that you can do it. Theoretically probably there are some assumptions involved which may or may not be right all the time and I think this point was brought out by the work presented here today so this puts the two in perspective.

CHAIRMAN COMAR Dr Bauer do you want to comment briefly? I don't think we can resolve this issue

DR. BAUER It seems that other workers[1, 2] have also made such calculations of accretion rates and the amount of exchangeable calcium.

CHAIRMAN COMAR Well I think the hour is getting late and the implications are understood. I would only like to comment on this matter of terminology admittedly it becomes confusing but I think we will agree that utter confusion is better than complete ignorance The thing about terminology is that one has to remember that expressions and terms are coined for the narrow purpose of the man who coins them and if we use them in that context for what good they will do, then I think we can get by But the minute we try to use them in the way we think they should be used or to use them in a broader context, then we get into difficulty

Also it is most useful whether or not the terminology is right to be able to relate an observation to the state of the patient and get correlations I think this is a very fine thing with or without bootstraps

On behalf of the panel and especially myself, I want to express appreciation to the people who planned the program I think it has been very fine so far and we are looking forward to even better things to come

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Part III

Ultrastructure of Bone

Chairman

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9
Fibrogenesis and the Formation of Matrix

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Introduction

The biosynthesis and morphogenesis of collagenous tissues occur in a graded series of stages, each involving a variety of activities these include the synthesis by the cells of the necessary macromolecules, which then become organized into precise arrays within the extracellular phase of the tissue The types of orientating forces which may be involved in tissue differentiation are not known, but these forces are exerted in such a manner that characteristic but very different tissue textures are formed, as seen, for example in cartilage and bone In certain systems, the final pattern appears to have been influenced by the initial spatial arrangement of the cells.

To enable comparisons to be made between the developmental patterns of the various tissues and to avoid confusion in terminology it is essential to stress that all collagenous tissues are composed of four similar phases (1) the cells—in developing tissues the synthesizing cells are usually referred to as fibroblasts, osteoblasts, or chondroblasts, but the general term of collagen-producing cells may be used [1] (2) the fibrous components, collagen reticulin, and elastin (3) the matrix or ground substance and (4) the interstitial fluid and free electrolytes. In bone and cartilage there is, in addition, the mineral phase In more detail, the predominant fibrous protein—collagen—gives a typical high-angle x-ray diffraction fiber diagram [2] has a characteristic amino acid composition which normally contains considerable proportions of glycine, proline and hydroxyproline as well as about 1 per cent hydroxylysine [3] and most fibrils demonstrate an axial periodicity of about 640 A [4] The matrix or ground substance surrounds the fibrous components and contains a mucopolysaccharide moiety which may itself contain several types of acid mucopolysaccharide mucoprotein,

noncollagenous protein collagen macromolecules not yet in fibrous form, and possibly small amounts of other unidentified substances These four phases occur in various proportions in the different collagenous tissues, and these variations are then partially reflected in the tissue structure which results

A fundamental assumption must be made that in each individual tissue the respective synthesizing cell is capable of producing both the fibrous protein and the material required for the formation of the matrix.[5] Cytochemical investigations of the cellular properties,[6-9] morphologic examination of the cellular organization,[5 10 11] and autoradiography of tissue sections[12 13] uphold this assumption Whether the cell synthesizes both moieties simultaneously or each moiety in alternating regulated phases, depending on the requirements of the particular tissue, is not known

It is necessary to try to construct a sequential and cohesive picture of the various activities and corresponding mechanisms that are being carried out during the process of fibrogenesis and the formation of matrix, particularly as they may apply to bone and cartilage The first part of this chapter therefore discusses some of the structural problems and types of mechanisms which may be involved in the initial formation of the individual components of the extracellular moieties, with special reference to the elaboration of the fibrous collagen protein Evidence in support of the mechanisms will be given In the second part, some results of a morphologic and biochemical investigation on the developmental structure of the organic material of avian long bones will be outlined and data related to the early mineralization of skeletal tissue will be considered.

Production and Aggregation of the Macromolecules

Two of the initial main stages involved in tissue differentiation are th chemical synthesis by the cells of the macromolecules required to forn the extracellular material and their subsequent aggregation and orientatio into extracellular components Some of the probable steps within thes stages will now be examined in more detail.

Structural Problems Associated with the Formation of Collagen Fibril In Vivo. CHAIN FORMATION Available data uphold that the collagen molecule is composed of three polypeptide chains arranged in a helical array about one another[14 16] It is not known whether the three chains have the same amino acid composition with or without variations in distribution along them or whether the amino acid composition of individual chains is different. During the synthesis of the chains the necessary amino acid residues must be brought together into their specific sequences and combined through peptide linkages to form the chains Nothing is known, however about the mechanism involved in the determination of the arrange

ment of the amino acid residues, nor what activating enzymes may be required in this process

A further structural problem is whether the three polypeptide chains are generated as single chains *A B C* and then spontaneously aggregated to form a macromolecular unit *ABC* presumably through some form of *H bonding or whether generation of all three chains to form a cohesive* unit *ABC* occurs simultaneously

HYDROXYLATION MECHANISM In collagen formation it is possible to follow by the use of radioactive isotopes in living tissue the synthesis of two characteristic amino acids hydroxyproline and hydroxylysine and their incorporation into the protein chains in a specific manner It has been established that in most collagenous tissues free hydroxyproline[17] and free hydroxylysine[18, 19] cannot be incorporated directly into the protein chains but that proline and lysine act as their respective precursors If this is true, the oxidation of proline and lysine must constitute an important step in the sequence of reactions which leads to the formation of the collagen macromolecule.

Stetten[17] has postulated that proline must be incorporated into a peptide linkage before hydroxylation can occur and a similar hypothesis has been proposed for lysine[20] Alternatively free proline (or free lysine) may be hydroxylated first and then incorporated into the protein chain The available chemical data on these reactions are complex and do not demonstrate conclusively which mechanism is responsible most workers have interpreted their results, however on the hypothesis that oxidation occurs after the formation of peptide linkages.[17, 20, 21] Such a mechanism implies that a proline-rich peptide destined for incorporation in the collagen molecule is first formed but attempts to isolate such a unit have so far been inconclusive[22, 23] On the other hand if oxidation were to occur nearly simultaneously with the incorporation of such an activated unit into the protein chains, the experiments may have failed to distinguish the presence of such free "activated" hydroxyproline Clearly the hydroxylation mechanism must influence the method of chain formation and possibly the size of the initial units

SITE OF SYNTHESIS WITHIN CELL Chemical analyses of fractions obtained after homogenization and differential centrifugation of collagen producing tissue cultures show that bound hydroxyproline is normally found within fractions of "large" particle size[24] In a simplified diagram of some of the results of this work the protein bound hydroxyproline content of four of the fractions is shown (Fig 9 1) It can be noted that with age most of the bound hydroxyproline appeared in the heavy fraction this fraction may be considered essentially as the extracellular phase but it also included the nuclei and adhering substance A considerable proportion of the bound hydroxyproline however was found in the large granule fractions, espe

cially in the younger cultures. These fractions are undoubtedly of cellular origin, and under the conditions of the experiments, the calculated particle size of the material contained within the second large granule fraction is about 3 000 A, a spherical shape being assumed Examination of sections of this material by means of the electron microscope showed that particles of varying internal structure were contained within this fraction.

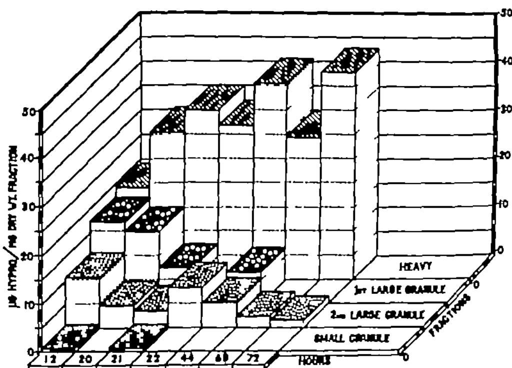


Fig 9 1 Diagram to show the amount of protein-bound hydroxyproline present in four fractions obtained from collagen-producing tissue cultures grown for various periods.

Furthermore the cultures were grown in contact with C^{14} L-proline for various times, and it was established that the radioactive proline was incorporated into a bound form in fractions of small particle size (about 250 A or less) It has not been possible to state however whether this proline-rich peptide material was in any way a precursor of the labeled hydroxyproline-rich substance found in the larger granule fractions.

It has been suggested that granules contained within the cytoplasm of collagen producing cells might have fibrogenic properties [8, 9, 23] and electron microscopy of various collagenous tissues in the avian embryo has demonstrated an abundance of cytoplasmic organelles in the collagen-producing cells [28] These structures are located in the identical position of the basophilic material Many sources of work have correlated these regions with protein synthesis thus the collagen protein may be synthesized in association with these organelles[5, 25] (Fig 9-4)

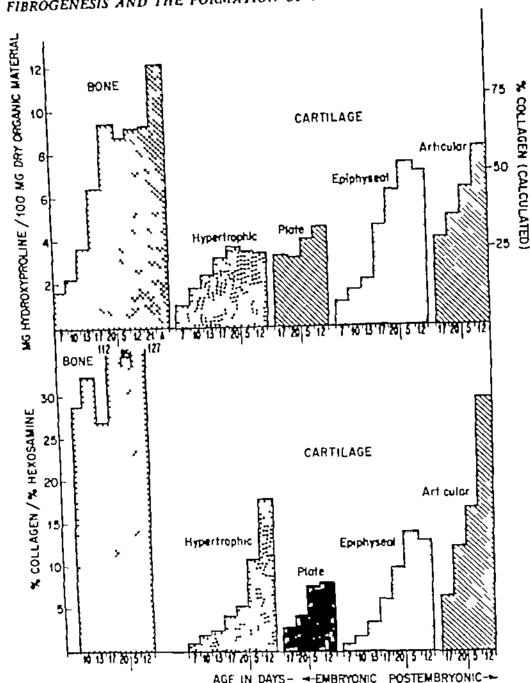


FIG. 9-2. *Top* Histograms to show the amount of protein-bound hydroxyproline present in five different regions of developing avian rudiments.

FIG. 9-3 *Bottom Histograms to show the ratio collagen content to hexosamine* content for the same five regions of developing avian rudiments

AGGREGATION OF MACROMOLECULAR UNITS A further structural problem to be considered is the method of aggregation of the macromolecules both longitudinally and laterally to form the first fibrillar units. Evidence obtained from precipitates of extracts obtained from fully formed collage

nous tissues led Gross, Highberger and Schmitt[28] to postulate the existence of a particle "tropocollagen" roughly 2 400 A in length. These units were believed to be capable of aggregating into specific fibrous patterns, the form of which depends on the agent of precipitation Neutral salts extracts of collagenous tissues were induced to form normal banded collagen fibrils simply by heating the cold solution to 37°C;[29] it was suggested that this fraction came primarily from the ground substance of the tissues and that the collagen units had not yet been in fibrillar form [30-32]

Recent physicochemical studies of solutions extracted from various collagens have shown the existence of particles of 3 100 A in length, 13.5 A in diameter and with a molecular weight of 360 000 These macromolecular particles behave in solution as rigid rods and confirm the hypothesis of tropocollagen units [33] Sonic irradiation of such particles in solution cleaves them into halves and quarters that retain the normal three-chain helical structure referred to above.[34] These findings imply that the molecular units of collagen could be about 770 A in length that is, close to the length of the repeating periodic structure seen in native collagen fibrils in tissues.

Bear[4] considers that the banded patterns observed within each period of the fibril arise from the lateral interaction between adjacent macromolecules and specific amino acid side chains He believes that the molecules are packed in almost perfect array in the interband regions, while the packing is less well ordered in the band regions and that they are partly composed of the more massive side chains Schmitt and his associates have suggested that the native collagen fibril is the product of aggregations of tropocollagen particles, all oriented in the same direction but staggered by specific lengths with respect to their molecular ends [35] From studies of the fibrous forms of precipitates obtained from sonic irradiated material, Hodge and Schmitt[36] have postulated that the longitudinal aggregation of the macromolecules may "involve a specific type of coiling of terminal dangling chains about each other" and that the bonding system would probably be composed chiefly of hydrogen bonds Gross[37] has stressed the importance of increased lateral bonding in mature fibrils On the other hand it has also been suggested that the small amount of reducing sugar which appears to be an integral part of the collagen fibril [38] may act as a stabilizing agent.[39]

It has yet to be shown, however whether the hypotheses derived from the studies of the mode of aggregation of tropocollagen particles outlined above can be applied to the method of fibril formation as it occurs in the tissues. It is possible that molecular units newly synthesized by the cell may differ in some respects, e g., in size or in side-chain arrangement, from the macromolecular particles obtained from the extracts, i.e., breakdown, of fully formed tissues. The evidence obtained from these studies is invaluable in providing an insight into the phenomenon of aggregation of collagen macromolecules in solution, but tissue fibrogenesis may require a more complex series of activities before aggregation can occur: these may include

the necessity of an activating agent, such as an enzyme which has not been found to be necessary in the experiments in vitro

SIZE OF PARTICLE AND SITE OF FIBRIL FORMATION The interrelationship between collagen producing cells and the extracellular components of collagenous tissues has been a subject of much debate, recent advances in technique have clarified the question to a certain degree. As a result of morphologic studies, Porter[11, 40] has suggested that a layer just beneath the cell membrane may become organized into collagen fibrils or that polymerization of definite fibrils may occur on the cell surface. Wassermann[41, 42] observed "primary or "microfibrils" at the surface as well as inside the cell while Fitton Jackson has stated that collagen fibrils mostly arise outside but in close association with the cell[5, 10] and that under certain specified conditions small groups of fibrils can arise within the cytoplasm.[43, 44] The last author stresses the dynamic nature of the cell surface and the fact that such a surface must be capable of permitting the passage of units of at least, macromolecular size[8, 25]

It is of interest to note that, in morphologic and biochemical studies of the culture system mentioned earlier banded collagen fibrils were not resolved by high-resolution electron microscopy in the tissues before 24 hours growth yet in this system the peak of hydroxyproline production had often occurred by that time[1] These findings led to the conclusion that "collagen fibrils are formed by the synthesis and secretion by the cell of a hydroxyproline-containing precursor of a protein or large peptide nature which itself has no banded structure in the electron microscope but which subsequently becomes directly transformed, without marked change in hydroxyproline content into banded fibrils" Fitton Jackson also concluded from other studies[5] that the transformation of the collagen molecular units into recognizable fibrils occurred on contact with the interstitial fluid the latter supplying a medium of appropriate ionic strength and pH whether the fluid also provided additional organic and/or enzymatic substances essential for the final formation of the fibrils was not known It is obvious that transformation into fibrillar form need not occur immediately in contact with the interstitial fluid, since individual fibrils, which are already clearly outside the cell increase in diameter with age subsequently to their initial formation

THE CRITICAL DIAMETER OF THE FIBRIL Most investigators have postulated that the collagen fibril when once formed increases in size by the accretion of further collagen molecules onto the periphery of the fibril.[10] Evidence obtained from the formation of tendon fibrils[8] has shown that the initial fibrils of any one bundle must have been formed almost simultaneously or by a very strictly controlled mechanism that, as the diameter of the fibril increased with age, there was a relative reduction of the interfibrillar material which surrounded each individual fibril and that the young developing tendon gave rise to a characteristic fiber diagram Hence

the succeeding molecules, as growth proceeds must be laid down on the fibrils in the form and packing appropriate to this x ray pattern

To enable such regulated growth to occur there must be a mechanism which permits the free passage of macromolecules through the interfibrillar regions until the local environment is appropriate and permits the further aggregation of the units onto the surface of a specific fibril or smaller unit. It has been pointed out that in collagen fibril formation the electric charge and its distribution on the molecule are primary factors in the phenomena of fibril formation [45] When a collagen molecule is passing through the interfibrillar milieu, the electric charges on that molecule and on the surface of adjacent fibrils must be in negative balance, and aggregation cannot occur but when the positive and negative charges on the molecular unit match those of an adjacent fibril aggregation occurs. From this it must be inferred that a substance within the interfibrillar material immediately surrounding certain fibrils may act as a temporary barrier or inhibitor to an aggregation mechanism Such a substance may be contained within the mucopolysaccharide moiety of the ground substance [46] As mentioned previously enzymatic action may also be required or may be involved in the above suggested mechanism. From this hypothesis a precisely balanced mechanism could be achieved and the environment of the individual fibrils within a bundle at any one time would be of fundamental importance.[3]

FIBRIL ORIENTATION TO FORM TISSUE FABRICS The spatial organization of the cells is such that they must have a general influence on the large scale structure of the respective tissue The orientation of the fibrils within each tissue is usually precise, the pattern often being one of single or multiple layers of fibrils arranged at certain angles with respect to each other [47–49] The sequence of events noted in the development of the basement membrane of amphibian skin, where an orthogonal layered pattern is obtained, indicated that the structural order must have been achieved by the "regrouping of units of lower but distinctly supramolecular order "[49] In earthworm cuticle individual layers of fibrils are laid down in turn at the cell surface each layer being orientated at 90° to the previous one [49] On the other hand, in developing tendon the fibrils are laid down in parallel array from the earliest stage and in bone the fibril alignment is such that adjacent fibrils are precisely opposed so that the banded pattern is in register The mechanism of such tissue arrangements is not yet understood

The Production of the Ground Substance. Many investigations have been made on the production of the ground substance and they cannot be detailed here One point of pertinent information was shown by Belanger [13] He demonstrated that the uptake of S^{35} initially occurred within the cartilage cells, an intense uptake being recorded within the first 2 hours of contact. Six days later however the radioactive sulfate was found to be exclusively in the extracellular areas Studies of dermal tissue culture[42] have

shown that they produce both hyaluronic acid and chondroitin sulfate but that sulfation was not fully completed

Developmental Structure of Avian Long Bones

The histogenesis of avian long bones has been described in detail by Fell.[50] She showed that the various morphologic regions in the cartilaginous rudiments are essentially homologous with those described in mammalian bones but that the developmental pattern of mineralization is different.[51] Investigations of periosteal bone[10–13] and of cartilage[52,53] in avian skeletal rudiments have shown that the characteristic collagen fibrils are present in newly formed bony tissue and in the articular cartilage of older embryos Most of the constituent fibrils of the remaining parts of the cartilaginous rudiment, however are not more than about 200 A in diameter and their periodic structure is not defined A further morphologic and biochemical study has been made of the interrelation of changes in the general chemical composition and the structural appearance of cartilage and bone during differentiation and growth of the tibial rudiment

Histologic Development. For clarity a description of some of the synchronous events in the development of avian long bone will be outlined On about the sixth day of embryonic life the periosteal bone begins to form by deposition of fibrous lamellae on the middle segment of the cartilaginous shaft The cartilage cells in the middle segment begin to hypertrophy at the same time erosion and resorption follow in about 4 days Calcification centers begin to appear adjacent to the periosteal bone in the hypertrophic zone on about the sixteenth day Endochondral ossification begins to appear in layers on the cartilage lining the marrow cavity in the 19-day embryo The zone of flattened cells which at first divided the hypertrophic zone from the epiphysis increases in depth in the 16-day embryo and may be compared with the epiphyseal plate of the mammalia The hypertrophic zone is equivalent to the meta and diaphysis In young chicks the articular and epiphyseal cartilages and the epiphyseal plate are quite well defined (Fig 9 5) the hypertrophic zone becomes fully calcified by about 12 days after hatching and the epiphyseal plate and epiphysis become narrower Independent ossification centers are present within the epiphysis.

Material and Methods. Sections of undecalcified rudiments of the tibia at different ages have been examined both by light and electron microscopy For correlated chemical analyses, a sufficient number of long bones, in a graded series of ages, were stripped of all extraneous connective tissue and dissected into five regions the periosteal bone, the hypertrophic cartilage the epiphyseal plate the epiphysis, and the articular cartilage Bone marrow and the areas of hypertrophic cartilage in which endochondral bone forma-







FIG. 9-4 *Upper* Electron micrograph of a transverse section of developing tendon in the avian embryo of 14 days. The intercellular areas are filled with immature collagen fibrils seen in cross section and are closely interlocked by the cytoplasm of adjacent cells (×20 000) (*Reproduced by permission of Proc Roy Soc London* *s.B* 144 556 1956)

FIG. 9 5 *Lower left* Histologic section of a 5-day chick bone rudiment to show the organization of the four regions of cartilage used in the work (×15)

FIG. 9-6 *Lower right* Electron micrograph of a typical cartilage cell from the central zone of the rudiment, before hypertrophy has begun. (×17 000)

tion had commenced were discarded The centers of ossification within the older epiphysis were also removed The present chapter presents some data about the amount of collagen present in different zones (based on hydroxyproline determinations) and the hexosamine content of the tissues The hexosamine content has been taken to represent the amount of mucopolysaccharide present in the respective tissues, since it is believed that the method of preparation of the tissues prevents contamination from other substances, such as plasma proteins, from contributing sufficient ex traneous hexosamine-containing material to invalidate the results.

The Fine Structure and Composition of the Organic Phase in Periosteal Bone. Present observations on sections of the developing bone of the tibia are essentially similar to those described for the tarsometatarsus of avian embryos[16, 23] Fibrogenesis occurs in intimate association with the osteoblasts, and collagen fibrils lie not only adjacent to but also merge with the surface of the cells The newly formed collagen fibrils show an axial periodicity of about 650 A, with a well-defined intraperiod structure The immediate appearance of such typical collagen fibrils adjacent to the syn thesizing cells is a characteristic of the formation of the organic material of bone

Results obtained from hydroxyproline determinations of the bone at different ages are shown in Fig 9 2 There was a rapid formation of bound hydroxyproline from the 7-day embryos to the day of hatching during the next 21 days however the content per sec remained fairly constant, but was less than that found in young adult birds. On the other hand, the amount of hexosamine was small averaging only 0 6 mg per 100 mg dry organic material. The ratio of the calculated collagen content to the hex osamine content is shown in Fig 9 3 and demonstrates the relative high density of the collagen phase in the organic material of bone.

The Fine Structure and Composition of the Organic Phase in Cartilage. HYPERTROPHIC CARTILAGE Prior to hypertrophy the chondroblasts are morphologically typical of collagen-producing cells in their internal struc ture (Fig 9-6) in addition dense homogeneous granules are sometimes present in the cytoplasm and may represent the glycogen moiety known to be present in the cells The cell surface is well defined, and thin processes extend into the surrounding intercellular spaces The extracellular phase is composed of numerous thin fibrils of not more than 200 A in diameter and occasionally showing a fine periodic structure of about 210 A The fibrils are randomly arranged (Fig 9 10) in relation to each other and are separated by considerable areas of low electron density In thicker sections the constituent fibrils appear to form a loose network Martin[52] has reported the presence of a few short fibrils showing normal periodicity in squash preparations of this region and noted that the fibrils had tapered ends

The amount of collagen is less than 2 per cent of the organic material in the 7-day embryo but there is a slow steady increase in amount during

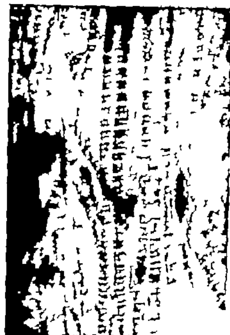








FIG. 9-7 *Upper left* Electron micrograph of the articular cartilage of a 5-day chick.

FIG. 9-8 *Upper right* The epiphyseal cartilage from the same specimen.

FIG. 9-9 *Lower left* The epiphyseal plate part of the extension of the cytoplasm from a flattened cell can be seen The same specimen

FIG. 9-10. *Lower right* The hypertrophic cartilage of the central region of the same specimen.

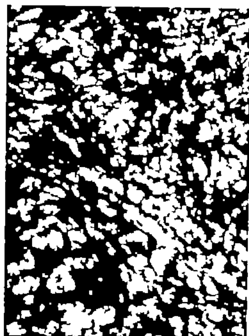
the next 10 days (Fig 9 2) subsequently the collagen content does not greatly exceed 20 per cent The amount of hexosamine is quite high in the 7 to 13-day embryos (about 8 mg per 100 mg dry organic material) It then begins to show a marked drop which is greatly accelerated in the young chicks, and finally the amount is only about twice that found in bone The ratio of collagen to hexosamine is shown in Fig 9-3 and depicts the sudden relative change in the proportions of the two substances in the young chicks

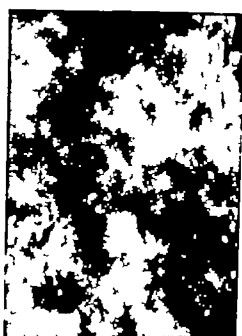
EPIPHYSEAL PLATE In thin sections, the extracellular material contains fibrils roughly 250 A in diameter which form a general honeycomb pattern, less dense material being present in the interstices. The processes of adjacent flattened cells in this area are often in contact with each other and the cells show an over all increase in the electron density as compared with those observed in the other regions of the rudiment (Fig 9 9)

The amount of bound hydroxyproline estimated as being present in the 17 and 20-day-old embryos is the same as that found in the adjacent hypertrophic cartilage (Fig 9 2) there is subsequently a slight rise in the content of collagen in the young chicks. The amount of hexosamine is higher in the epiphyseal plate in the 17-day embryos than in any other part of the cartilaginous rudiment at the same age This amount, however is equivalent to that found in the hypertrophic zone of much younger embryos Thereafter the hexosamine decreases in amount with age but is still considerably higher than that found in adjacent regions The ratio of collagen to hexosamine is shown in Fig 9 3

EPIPHYSEAL CARTILAGE The fibrils in the cartilage show the same type of honeycomb appearance observed in the epiphyseal plate but are arranged more densely (Fig 9 8) The hydroxyproline estimations show that there is rather a low rate of collagen formation in the 7 to 13-day embryos, the rate being similar to that in the hypertrophic zone Between 13-day embryonic and 5-day postembryonic life, however there is a rapid synthesis of collagen, the rate being the highest found in the various collagen regions (Fig 9 2) A slight drop then occurs which parallels the reduction in the area noted in histologic preparations The initial hexosamine content is typical and then falls to a lower level than that found in other regions Subsequently this level is maintained

ARTICULAR CARTILAGE. The fine structure of the articular cartilage is markedly different from that found in the other cartilaginous regions (Fig 9 7) It is composed of typical collagen fibrils arranged into bundles which are then formed into layers. Some small-diametered fibrils are also present as reported by Martin[52] The proliferating cells are more characteristic of fibroblasts than chondroblasts in their general shape the morphology and mode of formation of the extracellular phase are such that the articular cartilage is more comparable to the fibrous sheath of perichondrium sur





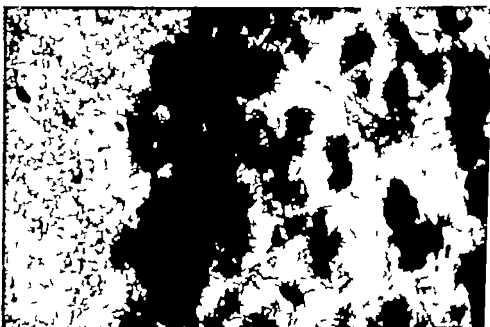


FIG. 9 11 *Upper left* Hypertrophic cartilage of a 5-day chick area immediately adjacent to the epiphyseal plate.

FIG 9-12 *Upper right* The amorphous substance appearing to mask the fibrils.

FIG. 9 13 *Lower* More fully calcified cartilage of a 5-day chick.

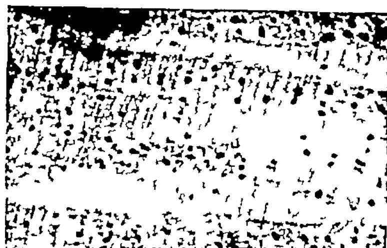
rounding the periosteal bone Indeed it may well be an extension of the perichondrium

The hydroxyproline determinations of this region indicate that the colla gen content per sec is slightly less than that recorded in the epiphysis of

the embryo but the curve is such that the general trend shows a steady increase in amount with age (Fig 9 2) On the other hand the initial hexosamine content is low compared with that found in the other regions of the cartilage rudiment and becomes further reduced with age The ratio of collagen to hexosamine shown in Fig 9 3 illustrates the predominant presence of collagen

GENERAL GROWTH TREND IN SELECTED AREAS The results from the chemical estimations outlined above show that the hydroxyproline content, taken as a measure of the amount of collagen protein present, parallels the histologic development of the long bone in the different zones of cartilage examined. The hexosamine content is more variable especially in the different zones of the cartilage examined the trend however is one of reduction in amount with age, the rate of loss being greatest in the areas where collagen synthesis is highest In the epiphyseal plate and in the epiphysis the ratio of collagen to hexosamine is much steeper than that found in the hypertrophic zone It is generally considered that these two regions are the chief proliferating zones in the rudiment, and the present figures would appear to support this contention and emphasize the over all influence of the collagen fraction It must be assumed that the fine fibrils observed by means of electron microscopy in the hypertrophic zone and in the epiphysis and the epiphyseal plate represent at least part of the collagen moiety even though characteristic collagen fibrils have not been clearly seen. It is also possible that some of the collagen protein present in the extracellular phase has not aggregated into fibrillar form perhaps because of the presence of an inhibiting substance referred to in the first part of this chapter The less dense material present in the extracellular areas presumably represents the ground substance

The Process of Mineralization. OSSIFICATION OF PERIOSTEAL BONE Robinson and his associates have made an extensive study by means of electron microscopy of mineralization in mammalian bone [54–57] The process seems to be essentially similar in avian bone[16] shortly after the organic matrix has been laid down by the cells, an amorphous matrix tends to mask gradually the newly formed tissue This phenomenon has been called the "calcification front." [55] It has been recorded that, in developing bone of the fowl embryo in the narrow area between two cells where collagen fibrils can still be clearly distinguished dense granules less than 100 A in diameter become precisely localized in a circular pattern between the *d* and the *ab* bands of each period of the collagen fibril (Figs 9 14 9 15) [55, 10] The particles were held to penetrate into the fibril to a certain degree and in an organized fashion electron diffraction has identified these particles as an apatite but no preferred orientation was apparent. Whether the precise localization was due solely to mechanical factors or whether a side-chain interaction occurred between the apatite and collagen fibril configuration was not known Robinson[57] has recorded the presence of dense







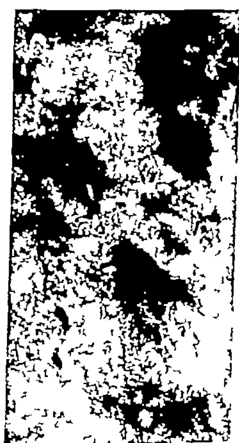


FIG. 9 14 *Upper left* Collagen fibrils of periosteal bone showing the dense apatite. (*Reproduced by permission of Proc Roy Soc London s.B* 146 270, 1957)

FIG. 9 15 *Upper right* Transverse section of one fibril to show arrangement of particles. (*Reproduced by permission of Proc Roy Soc London s.B* 146, 270 1957)

FIG 9 16. *Lower left* Hypertrophic cartilage showing the fine particles within the amorphous material

FIG. 9 17 *Lower right* A slightly later stage in calcificat the long crystals which have developed at of the

apatite particles within collagen fibrils of young mouse bone, they are some what similar in appearance to those observed in the avian bone

CALCIFICATION IN CARTILAGE Initial calcification of the 5-day chick tibia may be followed precisely in the cell layers of the hypertrophic zone lying adjacent to the epiphyseal plate. The sequence of events appears to be as follows the fibrous matrix becomes strongly honeycombed between the cells, which show signs of hypertrophying these cells at a slightly earlier stage had belonged to the lower layers of the flattened cells of the epiphy seal plate (Fig 9 11) An amorphous material then appears within the central compartments at some little distance from the cells (Fig 9 12) A very fine particulate material can be resolved within this substance as well as short fine needlelike particles each about 150 A long (Fig 9 16) These gradually increase in size until they measure about 1000 A or more in length the needles are pointed at one end and are randomly oriented but often arise from a central point and become radially arranged about this point (Fig 9-17) Finally a heavy calcified intercellular material is produced (Fig 9 13)

The content of hexosamine drops precipitously in the hypertrophic cartilage when calcification has started, whereas the collagen content re mains fairly constant It has also been noted that the adjacent epiphyseal plate contains the highest amount of hexosamine at a stage just prior to the onset of gross calcification in the hypertrophic zone These findings reflect similarities to those recorded in mammalian rudiments.[58, 59] Fur thermore the ratio of collagen to ash in the hypertrophic zone of young chicks is about the same as that found in periosteal bone of 17-day embryos

MODE OF CRYSTAL FORMATION The organization of the crystal phase in avian bone and cartilage shows certain differences when mineralization is about to occur an amorphous substance clouds the intercellular spaces but electron diffraction confirms the presence of apatite which at this stage may still be in an amorphous state. In subsequent ossification the localiza tion of apatite particles *in relation to one interband region of each period* of the collagen fibril in bone implies that the initial points of nuclei formation for the crystals occur here Once the nucleus has been seeded, further crys tal growth would occur spontaneously regularly arranged seeding points in association with the collagen fibril structure might also induce or influ ence the subsequent orientation of the crystal growth.[10] The use of x ray diffraction has demonstrated that the apatite crystals ultimately become preferentially oriented with their long axis parallel to the collagen fiber axis.[60]

In some elegant experiments Glimcher Hodge, and Schmitt[61] found that fibrils, precipitated from solutions of extracted collagen and showing the 640 A period ossified in the sense that apatite particles were identified in association with the precipitated fibrils. The particles lay within the fibrils, but were not located precisely in relation to one particular part of the

lagen fibril period as found in embryonic bone Collagen solutions precipitated in fibrous configurations other than the 640 A period did not ossify under the experimental conditions used. These findings stress the importance of the configuration of the molecular organization which produces the characteristic 640 A period in the process of crystal formation in bone.

A precise arrangement of initial apatite particles is not observed in cartilage Instead, during calcification fine dense particles, first present in an amorphous substance form into individual needlelike crystals which enlarge and become randomly arranged in the intercellular regions. If it is assumed that the fibrils present in the hypertrophic cartilage are composed of collagen molecules aggregated in the correct configuration, though the typical periodicity may be obscured, then precisely arranged seeding sites for *crystal nuclei could occur in association with these fibrils. Furthermore,* subsequent crystal growth would reflect two structural features present in the cartilage (1) the diameter of the fibril is relatively so small that few seeding points per period would be available (2) the known random arrangement of the constituent fibrils might influence the orientation of succeeding crystal growth Thus, the initial seeding mechanism for apatite in the two tissues may be identical but further development of crystal growth and alignment will reflect the original availability and arrangement of precise initiation sites within the collagen fibril period and the orientation of the fibrils.

The evidence for precise seeding points for crystal growth in relation to the fine fibrils in the hypertrophic cartilage is not available. Theoretically the mechanism discussed above could occur alternatively the process of calcification in cartilage might differ from the process of ossification in bone since both the structure and chemical composition of cartilage and bone differ in many respects.

Summary and Conclusions

The activities involved in fibrogenesis and the formation of matrix are numerous, and many may be as yet unknown. Seven successive steps, however which are probably concerned in the production and aggregation of the collagen macromolecules have been defined These steps may be regarded as the skeleton of the processes which are involved in the tissues during fibril formation Evidence drawn from both in vivo and in vitro experiments has supplied data which have been used to construct a more coherent picture of the possible mechanisms involved and is sufficient also to indicate that the defined steps may be regarded as distinct individual reactions. It must be stressed that the problems and mechanisms involved have been purposely stated in their simplest terms, but it is clear that more information is required before any of the mechanisms involved in these

steps in tissue fibrogenesis are understood and established as correct or are replaced by others

The examination of the fine structure and chemical composition of periosteal bone and four regions of cartilage in long bone rudiments has supplied data concerning growth at different histogenetic stages and has demonstrated certain variations which may be concerned in the mineralization of the tissue The morphologic development of apatite in bone and cartilage shows, respectively differences in formation which may reflect on the method of initial seeding of the apatite in relation to the component collagen fibrils in the two tissues

Acknowledgment

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10

Chemical Analysis and Electron Microscopy of Bone*

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Introduction

Efforts have been made by several investigators to correlate the chemical and histologic characteristics of calcified tissues in the human body Normally there are five calcified tissues in the human body enamel dentine, epiphyseal cartilage, bone and cementum At the present time the only distinctive differences which have been noted between the collagenous connective tissue matrices that calcify and those that do not are qualitative

Solomons and Irving[1] reported the results of their experiences in *1958* (1) By analysis ox hide ox achilles tendon rat tail tendon, ox dentine, human dentine, and ox bone all contain about 330 to 340 μmols of E amino groups per gram of protein (2) These E amino groups occurred on the lysine and hydroxylysine of collagen and not on other amino acids such as proline and glutamic acid where the amino groups form part of the peptide chain and are in other words in the A position. (3) By the fluorodinitrobenzine (FDNB) test, about 10 per cent of these E-amino groups are free in fully calcified dentine and bone, but after decalcification about 95 per cent of these are available to FDNB (4) In other collagenous matrices, by the FDNB test, with treatment with ethylenediammetetraacetate (EDTA) and with alterations of pH and the in vitro media, only 65 to 70 per cent of the E amino groups was available to FDNB After hydrolysis, about 95 per cent of these E amino groups was free or available to the dye

Therefore, it would appear that there is no great difference in the quantity of E-amino groups in soft tissue collagen as compared with hard tissue

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collagen as far as a weight percentage analysis of the collagen is concerned but that in the soft tissue collagen (even after EDTA treatment) only 65 per cent of these groups is available to the FDNB whereas in hard tissues after decalcification about 95 per cent of these groups is available When one of the latter tissues is calcified only about 5 to 10 per cent of the amino groups is available rather than 65 per cent as in the soft tissues prior to hydrolysis.

Follis[2] has recently shown that under the influence of aminopropionitrile or aminoacetonitrile a rat will produce epiphyseal cartilage that does not contain collagen in its fibril form but does contain collagen in its tropocol lagen or nonfibrillar molecular form Glimcher has recently shown that when the collagen is in this latter form it will not act as a nucleation site for apatite crystals in vitro but will do so when in the native (700 A) fibril form [3] Thus it would appear that cells may produce tropocollagen units but may not have the ability to organize these units extracellularly into fibrils. Again this appears to be a situation in which the physical organiza tion of the collagen rather than its chemical analysis on a weight per cent basis determines whether or not it will be associated with the process of calcification

In all the connective tissue matrices that calcify inside the epithelial confines of the body the inorganic crystals formed are those of hydroxyapatite so that there is no great qualitative difference in the basic composition of the inorganic material however the weight per cent of hydroxyapatite per gram of calcified matrix, including organic material varies in all five of the normally calcifying tissues.

The organic matrix in enamel is produced by an alleged derivative of the epithelial cell and is classified as a matrix containing keratin All the other matrices that normally calcify contain collagen and are produced by cells stemming from the mesenchyma. Despite this difference it is possible that the organic mechanism of inorganic crystal nucleation for calcification is the same in all these matrices

The term *matrix* is used to describe the extracellular material including fibrils and ground substance between these fibrils, in the five tissues that calcify This matrix may be more or less calcified The term *osteoid* is used to describe that matrix produced by osteoblasts

Enamel and Dentine

The only one of these five normally calcifying tissues which is relatively homogeneous—that is without admixture with cells or cell processes—is enamel Two studies should be noted that have been done on this tissue Weinmann, Wessinger and Reed [4] in 1941 tried to correlate the chemical and histologic analyses of developing enamel. In order to make such a correlation, it was necessary for them to consider the volume of the chemi

cally analyzed enamel in relation to its mass or weight. In other words, they tried to estimate the density of enamel matrix at various stages of development. They made "approximate estimations" on the developing enamel in the teeth of pigs. Later Deakins[8] investigated the relationship of the weight of the water organic, and mineral content of developing pig enamel with their volumes but he attacked the problem in a slightly different way than did Weinmann and his co-workers Deakins used small blocks of enamel of measurable volume He concluded that the water originally contained in the enamel matrix prior to its calcification was replaced almost volume for volume by the apatite and CO_2 inorganic components that eventually filled the enamel matrix during calcification The major postulate was of course that enamel matrix does not contract or expand during the calcification process. This appears to be a reasonable postulate in the case of all five of the normally calcifying connective tissue matrices.

Deakins findings varied from those of Weinmann, Wessinger and Reed in that the organic matrix did not seem to disappear significantly during the process of calcification, although in the graph in which he summarizes his findings there does appear to be a sharp drop in the milligrams of organic matter per cubic millimeter of enamel in the very first stage of calcification However shortly after calcification has begun, the quantity (or milligrams per cubic millimeter) of organic material in the organic matrix remains constant. During this process of calcification Deakins found that the wet pig enamel matrix increased in density from about 1 45 to 2.76 Berghash and Hodge[6] using a flotation method of determining the density of human enamel, found that in the dry state the density of sound permanent enamel which is approximately 96 per cent inorganic matter by weight, had a mean density of 2 95 to 2 96 with two maxima, one at 2.91 and one at 2 97 The total spread was from 2.86 to 2.98 and the maxima for sound deciduous enamel the sound part from carious enamel, and sound permanent enamel were between 2 96 and 2 97 Enamel contained about 7 per cent organic matter by weight in the case of sound deciduous enamel and the sound areas from carious teeth and about 4 per cent organic matter in the case of sound permanent enamel From these studies of this calcified matrix which is relatively homogeneous compared with other calcified tissues, we can obtain some information regarding the density of the dry inorganic matter containing a CO_2 component We note that, when this material is as fully calcified as possible It has in general a density when hydrated of about 2 76 and a density when dry of about 2 96 The picture obtained is shown in Table 10-1

Thus we see that even with the CO_2 component included in such an analysis pattern of enamel the density of the inorganic component is about 3 and certainly one would suppose that this would be true since even with the organic component included the density of the dry enamel when fully calcified approaches 3 that is, about 2.96 to 2.98 Unlike the other nor

Table 10-1

Components	*Weight*	*Density*	*Volume*
Water	3.7	1	3 7
Dry*	96.3	2.96	32.53
Organic (keratin)	(3.85)	(1 41)	(2.73)
Inorganic	(92.45)	(3+)	(29 8)
Total	100	2 76	36.23

This division of dry components into organic and inorganic is based upon the finding of Bergheah and Hodge that about 96 per cent of the dry weight in the permanent enamel is inorganic and about 4 per cent is organic The inorganic component includes the CO_3 associated with the apatite component of enamel.

mally calcifying matrices, the organic matrix of enamel is predominantly keratin instead of collagen

Enamel is the only one of the several normally calcifying connective tissue matrices that is fairly homogeneous that is, the calcified matrix is not intermixed anatomically with cells and cell processes Cementum,[7] dentine, bone, and even calcified epiphyseal cartilage contain cell and/or cell process spaces In the case of dentine these cell spaces have been studied by Feiler [8] He noted that the space occupied by the dentinal tubules in relation to the total volume of the dentine varied in different parts of teeth and in different age groups. Also the size of the dentinal tubules varied at different ages. For instance at the age of 10 years, he found that the cross-sectional area of the dentinal tubules was about 8 04 sq μ, at the age of 50 about 2 01 sq μ, and at the age of 75 about 1 13 sq μ. On the other hand the percentage of the volume of the dentine occupied by these cell processes, as represented by the tubules, decreased from the pulp to the dentinal-enamel junction occupying in the 10-year-old group about two-fifths (40 per cent) of the volume at the edge of the pulp cavity and only a tenth (10 per cent) of the volume near the dentinal-enamel junction Nevertheless it is seen from this study that dentine is not homogeneous and that there are two major divisions in this tissue namely the space occupied by the dentinal tubules and the odontoblast processes therein and the calcified collagenous matrix of the dentine surrounding the tubules Boyd, Drain and Deakins[9] found that the densities of 51 moist blocks of deciduous dentine were distributed around the mean density of 1 91 with a range of 1 64 to 2 09 while 91 slabs of moist permanent dentine had a density of 2 0 with a range of 1 71 to 2.23 Manley noted the density of dry dentine to be 2 1 to 2 33 but he finally concluded that, since he was using a refractive index method dependent on submerging a specimen in a fluid medium in the dried specimens some of the dentinal tubules were probably filled with air so that the true density of the calcified matrix would probably exceed the maximum of 2.33 which he found [10]

Importance of Specific Gravity or Density in Correlating the Chemical Analysis and Anatomy of Bone Specimens

It became obvious from investigations on dentine and enamel that the correlation of the chemical analysis of bone specimens with their anatomy for practical purposes must be based on their density the mass or weight per unit volume The reason is that microscopic anatomic analyses are usually associated with relative volumes, whereas chemical analyses are usually associated with weight per cent Density being the ratio of mass or weight/volume correlates these two types of analysis.

Three factors must be taken into account in bone analysis (1) the over all mass per unit volume or density of the whole specimen (2) the density of its constituents, and (3) the fact that the relative amounts of each of its constituents may vary simultaneously This complexity which Deakins recognized in enamel is multiplied twofold in bone cementum, dentine and epiphyseal cartilage by the fact that two major parts are present in these tissues, each of which may have variations in over all density the highly hydrated cellular system and the less hydrated calcified collagen matrix

"Where these conditions obtain the usual percentages of weight analyses are inadequate and misleading," Deakins stated. "For example on a per centage basis an increase of inorganic material having a high density [3 for instance] automatically results in an apparent decrease of organic mat ter [density 1 41] whether the specific amount (i.e milligrams per cubic millimeter) changes or not. This difficulty can be avoided by referring all *determinations to volume instead of dry weight. A unit volume is constant*"

However to make such a system understandable, one must know what type of density determination is being made and, in the case of bone, how *the two parts of this tissue and their densities intermix to* produce the over-all density of the tissue when it is hydrated and when it is dry It is particularly important to understand this concept when dealing with osteoporotic bone Otherwise, chemical data may be interpreted as showing osteomalacia when in fact the actual bone substance in a porotic bone may be just as "fully calcified" as in "normal" bone which is not porotic.

DENSITY OF WHOLE BONE SPECIMENS

The specific gravity and thereby the density of bone specimens has been a subject of recorded interest for over 100 years. Wertheim (1847) [11] Aeby [12] Rauber (1876) [13] Krause and Fischer [14] Hulsen (1898) [15] Tsai and Lin (1939) [16] Mack (1950) [17] Evans, Coolbaugh, and Lebow (1951) [18] Amprino (1952) [19] Gillespie (1954) [20] and Robinson and Elliott (1957) [21] have published data on this subject. Deakins (1942) [5]

C Dry (There are several drying methods, and with these methods different drying times have been used by various investigators.)
1 Excluding the water space
2 Including the water space after drying

III Bone density as measured on a piece of bone stripped of medullary marrow and periosteum
A Fully hydrated
1 In distilled water
2. In normal saline
B Moist (cf. remarks under II*B*)
C Dry (cf remarks above under II*C*)
1 Excluding the water space (Such a density determination is governed by three factors the amount of collagenous osteoid matrix, the amount of hydroxyapatite mineral crystalline material and the amount of organic matter in the marrow vascular-osteocyte space)
2 Including the water space (from which the water has been removed and which is then occupied by air or a vacuum in dried specimens) ‡

IV Density of a microscopic portion of calcified bone matrix, excluding marrow vascular space
A Hydrated
1 Including canalicular and lacunar volume.
B Dry
1 Including canalicular and lacunar water space
2. Excluding canalicular and lacunar water space (The packing of collagen fibrils in a unit volume of osteoid matrix varies somewhat in adjacent bone lamellae,[24] and there are apparently differences in the average collagen concentration per unit volume in average osteoid matrix of different animal species When the collagen per unit volume of osteoid is less, there would

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‡ Evans, Coolbaugh and Lebow[34] reported experiments in which the density of bone samples was calculated from measurements of β-ray transmission through pieces of *dry* cortical bone from a Sr^{90} source. In general the percentage of β-ray transmission is proportional to the logarithm of the material's density The density values they obtained were more typical of those obtained by other investigators who used *hydrated* cortical bone rather than dry bone. It would appear that the density values obtained by Evans et al. published in 1951 were not corrected for the porosity of the specimens. All bone specimens, even the most compact ones are porous to some degree. Therefore, thin specimens free of water would have had air occupying the osteocyte spaces at least. Since air has a lesser density than water one must assume that the density figures they obtained were a little less than those which would have been found if their specimen had been hydrated and less than those which would have been obtained if correction had been made for the water space filled by air in their material. They found that the average density of the cortex of human bones which they investigated was as follows femur 1.9- tibia, 1 92, and fibula 1.89

appear to be more room for bone crystals Thus the density of such material as this at this microscopic level of organization is largely a function of the reciprocal relationship between two factors the amount of collagen and the amount of hydroxy apatite per unit volume of calcified bone matrix)

The author has used two of these four types of bone density determinations in his work IIIA1 which will be called D^{A} and IIIC1 which will be called $D^{C(b)}$ as contrasted with IIIC2, which will be called $D^{C( )}$ Actually specific gravity measurements were made, and these could be converted to density figures [1]

RELATION OF CHEMICAL ANALYSIS TO DENSITY OF BONE SPECIMENS

The inorganic component including the "CO_2 space" was calculated by Robinson and Elliott to be about 2.89 The density of the dry inorganic component which includes the apatite crystals plus the CO_2 and the other inorganic components such as sodium, potassium chloride and also calcium and phosphorus in a noncrystalline state (taken from the CO_2 space) is calculated to be about 3 0 The density of the dry organic component (using type III bone density determination) is about 1 45 when separated from calcified bone by EDTA decalcification but is 1 41 theoretically[23] and also when the organic matter of the CO_2 space is extracted from that space and included with the organic fraction The density of water is 1 Fat can replace water in the marrow vascular space and fat has a density of about 0 9[28] Table 10-2 demonstrates average figures obtained on samples of dog bone tibiae[21] The figures in columns 1 to 14 are those obtained when the analysis pattern of these periosteum and endosteum-free bone specimens included the following divisions water inorganic, organic, and CO_2 space[21] The figures in columns 15 to 20 are those obtained when the CO_2 space was divided as outlined above between the inorganic and organic components so that only three divisions remain water inorganic, and organic §

The figures in Table 10-2 columns 1 to 14 were derived by constructing a sloping line on a graph through the data points[21] The direction of this line was determined by the method of least squares, using data from 228 bone specimen analyses The figures are therefore those found on such lines, relative to (1) the density of the bone samples when fully hydrated and (2) the weight per cent contributed by each division of the analysis pattern[21] Figures derived in this way have one disadvantage the line is that which most nearly approaches all the points, so that if a curve occurs in the data distribution the whole line may change its slope but the line will

§ This alteration or redistribution of the CO_2 space does not change the over-all density of the various analysis patterns (cf column 21)

Table 10-2

AVERAGE FIGURES ON STRIPPED DOG BONE TIBIAE

CO_2 space separate														CO_2 space divided						
Density of hydrated bone (1)	Wt of hydrated bone Gm (2)	Vol of hydrated bone cm³ (3)	Wt per 100 cc water (100°C dried) (4)	Wt of dry bone Gm (5)	Vol of dry bone cm (6)	Wt (D 1 46) of organic (7)	Vol of organic (dry) (8)	Wt of (D 2.80) ash + CO_2 space (9)	Vol of ash + CO_2 space (10)	Wt of bone ash (600°C) (Density 3.00) (11)	Vol of bone ash (12)	Wt of CO_2 space (D 2.08) (13)	Vol of CO_2 space (14)	Wt and vol of water (D 1.0) (15)	Wt of dry (16)	Wt of inorganic (D 3 0) (17)	Vol of inorganic (18)	Wt of organic (D 1 41) (19)	Vol of organic (20)	Dry density (21)
2.1	100	47.62	8.1	91.0	39.59	22.5	16.52	69.4	24.00	60.0	10.00	8.5	4.1	8.1	91.0	68.20	22.75	23.04	16.77	2.325
2.05	100	48.78	10.1	89.9	38.08	22.1	15.24	67.8	23.14	59.5	19.45	8.3	3.90	10.1	89.9	66.72	22.24	23.18	16.44	2.3243
2.0	100	50.00	12.2	87.8	37.80	21.6	14.90	66.2	22.90	58.1	19.00	8.1	3.0	12.2	87.8	65.10	21.70	22.70	16.10	2.323
1.9	100	52.63	16.7	83.3	35.03	20.7	14.28	62.6	21.65	55.0	17.00	7.0	3.60	16.7	83.3	61.58	20.53	21.72	15.40	2.318
1.8	100	55.56	21.7	78.3	33.80	19.7	13.50	58.6	20.27	51.5	16.83	7.1	3.44	21.7	78.3	57.60	19.22	20.04	14.64	2.313
1.7	100	58.82	27.3	72.7	31.52	18.5	12. 0	54.2	18.70	47.6	15.58	6.6	3.18	27.3	72.7	53.32	17.77	19.38	13.75	2.307
1.6	100	62.50	33.6	66.4	28.00	17.2	11.86	49.2	17.04	43.2	14.13	6.0	2.91	33.0	66.4	48.40	16.13	18.00	12.77	2.298
1.5	100	66.67	40.8	59.2	25.87	15.7	10.83	43.5	15.04	38.2	12.40	5.3	2.55	40.8	59.2	42.87	14.29	16.33	11.58	2.288
1.4	100	71.43	49.0	51.0	2?.13	14.0	9.66	37.0	12.77	32.5	10.62	4.5	2.15	49.0	51.0	36.55	12.18	14.45	10.25	2.274
1.35	100	74.07	53.5	46.5	20.57	13.1	9.03	33.4	11.54	29.4	9.01	4.0	1.93	53.5	46.5	33.01	11.00	13.40	9.57	2.261
1.3	100	76.92	58.4	41.6	18.53	12.1	8.34	29.5	10.18	26.0	8.5	3.5	1.68	58.4	41.6	[illegible]	9.74	12.38	[illegible]	2.240

remain a straight line and will not curve to fit the data points Thus we find that the data on very porous bone samples which have a low density when hydrated do not fit this table so well as we should like However in the upper range of hydrated densities these data points are very reproducible, and so the bone specimen analyses are more uniform in bones having a density of 1 7 to 2 05 when hydrated than in those with a lesser density

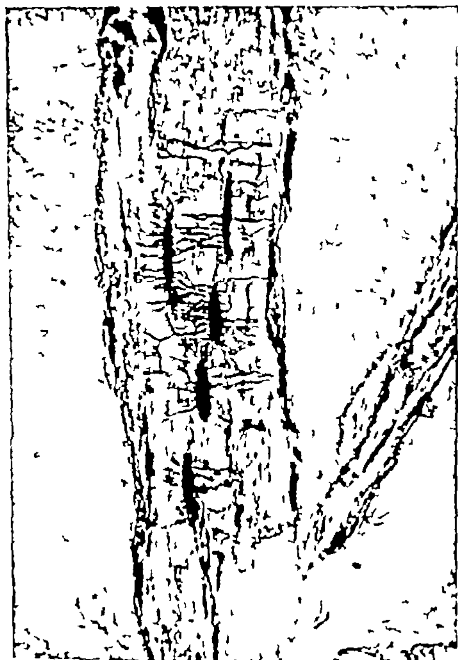


FIG 10-1 Fragment of cortex of human rib autoclaved at 27 lb pressure for 2 hours. Sprayed with a viscid plastic so that air remained in the canaliculi and lacunae. Viewed in the light microscope (×500) (*Reproduced by permission of J Bone & Joint Surg., 34A 398 1952.*)

Marrow-Vascular Osteocyte Space Characteristics. In bone one has Haversian canals which we may call marrow vascular spaces. Ham[77] pointed out that none of these canals in a dog s radius lay more than a distance of about a quarter of a millimeter from any cell in the bone and *most cells were within 0 1 mm of a blood vessel For purposes of analysis,* particularly the major water space one must deal with a marrow vascular osteocyte space in other words the Haversian plus the canalicular and lacunar space in the bone sample

By our measurements on histologic sections, cells and cell processes in



FIG 10-2. Part of an osteocyte. The word "cell" lies over the nucleus. The cytoplasm and the cytoplasmic extensions are seen peripheral to the nucleus. The space between the cytoplasm and the matrix is considered to be an artifact where the cytoplasm and cell membrane have pulled away from the matrix. The matrix in this specimen was decalcified The arrows point to protoplasmic extensions of the cytoplasm extending out through the matrix in the canaliculi. (Approximately ×33 000) *(Reproduced by permission of J Bone & Joint Surg 40A, 689 1958)*

lacunae and canaliculi occupy 10 to 15 per cent of bone matrix volume while the volume of the whole specimen occupied by the marrow vascular space varies widely (Figs 10-1 10-2)

By analysis bone samples having the *least* organic and inorganic matter (dry substance) per unit volume contain the most water [21] If the chief mechanism in the bone specimens such as we used for holding water had been the *osteoid* matrix or the surfaces of the apatite crystals in the osteoid matrix, then more water might be expected to have been found in those bone specimens which per unit volume contained more of these dry substances (Fig 10-3)

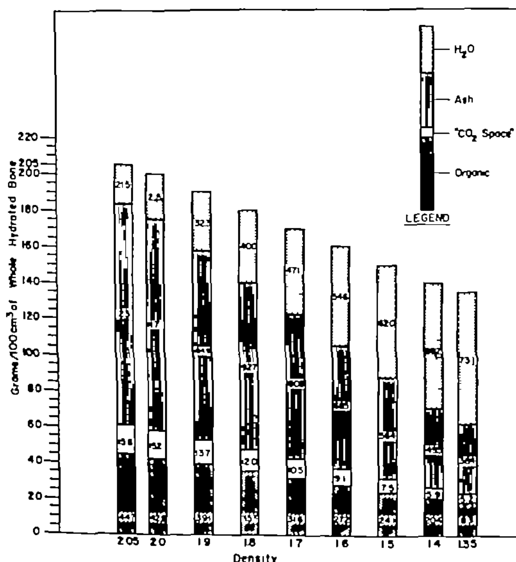


Fig 10-3 A bar graph showing results of bone analyses. It will be noted that the water content of specimens having lower specific gravities or densities when hydrated is greater in proportion to the solids in the specimen than in the case of those bone specimens having a higher specific gravity or density when hydrated

Marrow Vascular-Osteocyte Space Characteristics. In bone one has Haversian canals which we may call marrow vascular spaces. Ham[77] pointed out that none of these canals in a dog's radius lay more than a distance of about a quarter of a millimeter from any cell in the bone and most cells were within 0 1 mm of a blood vessel. For purposes of analysis, particularly the major water space one must deal with a marrow vascular osteocyte space in other words, the Haversian plus the canalicular and lacunar space in the bone sample

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If the bulk of the water in hydrated bone is *not* retained by the calcified osteoid (extracellular matrix of the bone cells) when the osteoid is normally calcified, then it must be retained by the marrow and bone cells and the extracellular noncalcifying organic matrix in the marrow vascular space.

In the dry state the inorganic components of bone have a density (about 3) which is about twice as great as that of the organic ones (about 1.41). The similar density of the dry bone specimens implies that the *ratio* between the two most prominent solid components in a bone specimen (namely the inorganic apatite crystals and the collagen fibrils of the bone matrix or osteoid) must be similar regardless of the amount of water the specimen retained in its marrow vascular-osteocyte space before drying.

Actually there is a tendency for the density of the dry specimens if of sufficient size for accurate measurement from very young animals to fall off a little. This is understandable for two reasons.

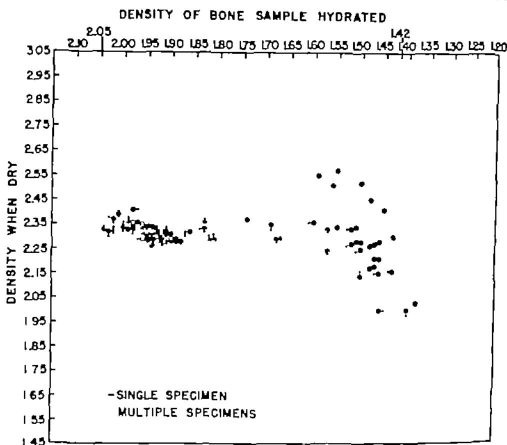
1. In a very porous bone specimen the density when dry will be a little less than that of a more compact bone (having osteoid of comparable percentage calcification) because there will be a larger marrow vascular osteocyte space in the specimen in relation to the space occupied by the calcified matrix. Then a larger percentage of the dry material in the specimen will be dry organic matter ($D \approx 1.41$) belonging to the stroma and cells of the noncalcifying marrow vascular-osteocyte space and a smaller percentage of the dry matter will be contributed by the calcified osteoid matrix ($D \approx 2.3$).

2. In a newborn animal in which a larger percentage of the osteoid matrix contains water rather than inorganic crystals, or in other words when over-all matrix calcification is not so "full" or "complete"[19] the dry density will be further decreased because the dry density of the calcified osteoid will drop toward $D \approx 2.0$ from $D \approx 2.3$.

Marrow is a very cellular tissue and cells have a high water content. Barer and Joseph[28] found that the extreme range of cell water content (weight per cent) is 84 to 90.4 per cent, and they stated that, "the assessed value of 88 per cent is unlikely to be much in error." The cells in a bone specimen are a known depot for water.

The greatest single variant in marrow anatomy appears to be due to the replacement of water in the marrow cells by fat. In general the density of water is 1 and that of fat is 0.9 although slight variations in this figure of 0.9 are found in various animal species.[20] Dog bone such as we used had a minimum of fat. However once adjustment is made for this variable ||

|| *Defatting in relation to hydrated bone volume weight and density.* In practice the specimen is defatted, the loss of weight due to defatting is divided by 0.9 which gives an equivalent water weight. The difference between the water weight and the fat weight is then added to the predefatting weight of the hydrated specimen. This sum gives the weight the specimen would have had if it had originally contained a volume of water equal to the volume of fat it actually contained. Similarly the



BONE DRIED AT 100°–105 C IN AIR FOR 8 HOURS AFTER CLEANING AND FULL HYDRATION (488 SPECIMENS)

FIG 10-4

Therefore, it was of interest to find[31] that the average specific gravity and density of 228 *dry* macroscopic specimens of puppy and dog bones was quite constant and averaged 2 34 ± 0 13 even though these same bone samples had demonstrated a wide variation in density (2 05 to 1 35) when hydrated Only in 28 samples of bone from newborn puppies was the average dry density 2 23 ± 0 14 In 139 specimens, only from adult dogs, it averaged 2 35 ± 0 05 These specimens were dried at 100 to 105 C in air for dry density determinations $D^{d(b)}$ (Fig 10-4)

The logical conclusion drawn from our finding that dry bone from most normal canine sources has a similar density is that the bulk of the dry substance in a whole bone specimen (even from newborn animals) has a fairly similar composition This dry substance of fairly uniform average composition (but not absolutely the same composition) can somehow in a hydrated bone specimen be associated with more or less *water* (which has a density of 1 at 4 C) Various amounts of dry bone and water are combined to yield the widely varying densities of fully hydrated bone specimens.

If the bulk of the water in hydrated bone is *not* retained by the calcified osteoid (extracellular matrix of the bone cells), when the osteoid is normally calcified then it must be retained by the marrow and bone cells and the extracellular noncalcifying organic matrix in the marrow vascular space.

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Actually there is a tendency for the density of the dry specimens if of sufficient size for accurate measurement from very young animals to fall off a little. This is understandable for two reasons

1 In a very porous bone specimen the density when dry will be a little less than that of a more compact bone (having osteoid of comparable percentage calcification) because there will be a larger marrow vascular osteocyte space in the specimen in relation to the space occupied by the calcified matrix Then a larger percentage of the dry material in the specimen will be dry organic matter (D^{4}1 41) belonging to the stroma and cells of the noncalcifying marrow vascular-osteocyte space and a smaller percentage of the dry matter will be contributed by the calcified osteoid matrix (D^{4}2.3)

2. In a newborn animal in which a larger percentage of the osteoid matrix contains water rather than inorganic crystals, or in other words when over all matrix calcification is not so "full" or "complete"[19] the dry density will be further decreased because the dry density of the calcified osteoid will drop toward D^{4}2 0 from D^{4}2 3

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|| *Defatting in relation to hydrated bone volume weight and density* In practice the specimen is defatted, the loss of weight due to defatting is divided by 0.9 which gives an equivalent water weight. The difference between the water weight and the fat weight is then added to the predefatting weight of the hydrated specimen This sum gives the weight the specimen would have had if it had originally contained a volume of water equal to the volume of fat it actually contained. Similarly the

buoyancy which is represented by the difference between the water and fat weight is lost by defatting so the submerged weight would also increase by the same amount. Example

Weight of *hydrated* bone specimen in air		Weight of hydrated bone submerged in water		Volume of hydrated bone specimen
1 000 mg prior to defatting +10 mg gained during defatting	−	500 mg prior to defatting +10 mg gained during defatting	=	500 mg prior to defatting and 500 mg after defatting
1 010 mg *after* defatting		510 mg after defatting		

The hydrated specimen volume should not change but the specific gravity and density of the specimen would change by defatting

$$\frac{M^h}{V} = D^h$$

$$\frac{1\,000}{500} = D^h 2.0 \text{ before defatting} \qquad \text{and} \qquad \frac{1\,010}{500} = D\ 2.02 \text{ after defatting}$$

Defatting in relation to dry bone volume weight and density Removal of fat will reduce the dry weight measurement made prior to defatting. This reduction will be equal to the weight of the fat removed unless the defatting method inadvertently removes significant amounts of other organic and inorganic matter from the specimen in addition to fat. Example

Weight of dry bone specimen in air		Weight of water displaced by *dry* bone volume = X		Weight of *hydrated* bone specimen submerged in water
880 mg before defatting −90 mg lost during defatting	−		=	500 mg prior to defatting +10 mg gained during defatting
790 mg defatted weight = A				510 mg after defatting = B

$(A - B = X)$	A	B	X
Before defatting	880 mg	500	380 mg
After defatting	790 mg	510	280 mg

Therefore density of *dry* bone specimen *before* defatting

$$\frac{M^d}{V^d} = D$$

$$\frac{A}{B} = \frac{880}{380} = 2.31$$

and density of *dry* bone specimen *after* defatting

$$\frac{A}{B} = \frac{790}{280} = 2.82$$

it appears that an approximately standard marrow pattern may be constructed Huggins et al [29] found that the fat free solids constitute about 20 per cent of the weight of marrow of rabbits Krause[30] concluded that "an inverse relationship (exists) between the water and the lipid content of marrow" Extending the curve on his graph one might find about 85 per cent water and 15 per cent solid residue in the theoretical situation wherein no fat would be present in the marrow of cats When fat is present then *the water space in marrow* is intruded upon by this fat, as pointed out by Krause,[30] Dietz,[31, 32] and Dietz and Steinberg [33]

Another way of arriving at a standard marrow pattern is to consider the obvious fact that red marrow is a very cellular tissue and is comparatively fat free The water content based on the average water content for cells given by Barer and Joseph[25] would suggest that 88 per cent of the total weight of marrow fat free was water and 12 per cent was dry solids. This compares well with the figure of 85 per cent and 15 per cent obtained by an extension of the line on the graph of Krause [30]

Hazen[34] determined the inorganic ash content of both fatty and red bone marrow in the human. Since we are proposing use of a standard fat free *marrow for this work* his data for red bone marrow will be used 0.554 per cent of the total hydrated marrow weight was inorganic matter For simplicity the figure of 0 6 per cent is used here

Throughout this work a figure for the density of organic material is 1 41 which is the theoretical density of dry collagen the density of the inorganic matter (including CO_2)** is about 3 the density of water is 1 Using these various figures the standard marrow pattern was derived and is shown in Table 10-3

Table 10-3

STANDARD MARROW PATTERN

Components	*Basis of grams per 100 Gm*			*Basis of cm³ per 100 cm³*		
	Weight	*Density*	*Volume*	*Weight*	*Density*	*Volume*
Water	88 0	1 0	88 0	91 40	1 0	91 40
Inorganic	0 6	3 0	0.2	0 62	3 0	0.20
Organic	11 4	1 41	8 09	11.84	1 41	8.40
Total	100	1 04	96.29	103.86	1 04	100.00

Thus the standard marrow (fat free) is calculated to have D^{t}1 04 D^{m}0 12, D^{o}1 45 Water content by weight, 88 per cent. Water content by volume, 91 4 per cent

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** Inorganic density is based on the finding that the theoretical value for apatite is 3 12 to 3 15[illegible] whereas the inorganic density when combined with the CO_2 is somewhat less and as pointed out above in reference to previous studies on enamel is probably around 3 (cf Table 10-2)

buoyancy which is represented by the difference between the water and fat weight is lost by defatting so the submerged weight would also increase by the same amount. Example

Weight of *hydrated* bone specimen in air		Weight of hydrated bone submerged in water		Volume of hydrated bone specimen
1 000 mg prior to defatting +10 mg gained during defatting	−	500 mg prior to defatting +10 mg gained during defatting	=	500 mg prior to defatting and 500 mg after defatting
1 010 mg *after* defatting		510 mg after defatting		

The hydrated specimen volume should not change but the specific gravity and density of the specimen would change by defatting.

$$\frac{M}{V^h} = D^h$$

$\frac{1\,000}{500} = D^h 2.0$ before defatting and $\frac{1\,010}{500} = D^h 2.02$ after defatting

Defatting in relation to dry bone volume weight and density Removal of fat will reduce the dry weight measurement made prior to defatting. This reduction will be equal to the weight of the fat removed unless the defatting method inadvertently removes significant amounts of other organic and inorganic matter from the specimen in addition to fat. Example

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FIG 10-6 Full thickness cortex of the dog's tibia. This specimen was from a younger animal than that shown in Fig 10-5 Its specific gravity or density when hydrated was about 1 85 (Approximately ×200) (*Reproduced by permission of Clin Orthop 9 27 1957*)

in canaliculi and lacunae Of the area occupied by the bone matrix, 10 per cent is supposed to be occupied by the canaliculi and lacunae By adding the 10 per cent figure to the area measured by planimetry and weighing methods for the marrow vascular space in each specimen it was possible to calculate the total volume occupied by the marrow vascular-osteocyte space in each specimen. Of this space 91 4 per cent is water according to our calculations, or 88 per cent of the weight of this space is due to water By heating the specimens at 50 C, a close correlation was obtained between the amount of water calculated in each specimen in the marrow vascular osteocyte space and that removed from the specimen before decalcification and sectioning by 50°C drying A little extra water is removed when the specimen is heated above the shrinkage temperature of collagen at 100 C, as will be seen in Table 10-4 This extra water removed between 50 and 100°C is therefore assumed to be water derived from the calcified matrix region of the fresh bone specimen.

We have also noted that, in those cases where the bone was heated to 100 C before decalcification in an EDTA solution, the bone disintegrated Whereas when water was removed by 50 C drying or when it was not removed prior to decalcification, such disintegration of the bone sample did

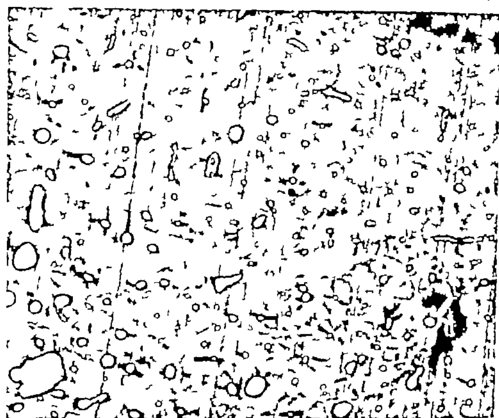


FIG 10-5 Full thickness transverse section of the tibial cortex, midshaft of the dog. This specimen had a specific gravity or density when hydrated of about 2.05 (Approximately ×200) (*Reproduced by permission of Clin. Orthop* 9 28 1957)

In the case of bone specimens it has been noted that the bulk of water is removed when the tissue is dried at 50 C and a smaller amount of water is removed when the drying temperature is increased from 50 to 100 C. It would appear that the water that can be removed at 50°C is less tightly bound to the bone specimen than is water that is released at 100°C. It is interesting in this regard to see that the water removed at 50 C about equals the amount of water which can be calculated to be in the marrow-vascular osteocyte space in a specimen on the basis of photomicrographs of sections of the specimen The water removed between 50 and 100 C must then appear to come out of the calcified bone matrix (or calcified osteoid) of the bone sample (Figs. 10-5 to 10-7)

Note the increasing area occupied by the marrow vascular spaces in Figs. 10-5 to 10-7 In this work it has been concluded that the marrow vascular and osteocyte spaces had a water content of 91 4 per cent by volume and 88 per cent by weight. The section thickness of all three specimens is about the same Therefore a measurement of areas occupied by marrow vascular spaces in each of the three specimens subtracted from the total area measured gives the area occupied by the bone matrix and cells



FIG. 10-6 Full thickness cortex of the dog's tibia. This specimen was from a younger animal than that shown in Fig 10-5 Its specific gravity or density when hydrated was about 1 85 (Approximately ×200) (*Reproduced by permission of Clin Orthop., 9 27 1957*)

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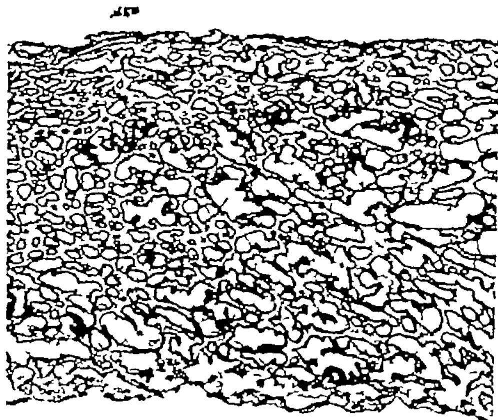


FIG. 10-7 Full thickness cortex of the tibia of a puppy under 6 weeks of age. This specimen had a specific gravity or density of about 1.35 when hydrated. (Approximately ×200.) (*Reproduced by permission of Clin Orthop., 9 27 1957.*)

not occur during EDTA decalcification. Thus it would appear that the water removed between 50 and 100 C was associated in some way with the structural integrity of the collagen matrix of the bone. Finally we note that the shrinkage temperature of bone collagen after EDTA decalcification is between 55 and 70 C. It appears that there is one rough way of approximating the amount of water in each of the two major anatomic divisions of a bone sample that water which is removed at 50 C drying below the T of bone collagen represents largely the water associated with the marrow-vascular-osteocyte space whereas the water removed subsequently at 100 C drying represents the water mainly associated with the calcified organic bone matrix (Figs. 10-8 10-9)

The following four factors are illustrated in Fig 10-9

1° Below Shrinkage Temperature of Collagen After this drying temperature, compact bone of adult dogs does not disintegrate during subsequent EDTA decalcification. However porous bones of very immature puppies do show some disintegration even after only 50°C drying Such bone is not only more porous, but its calcified osteoid has less over-all calcification as is typical of the calcified bone matrix of very young animals.

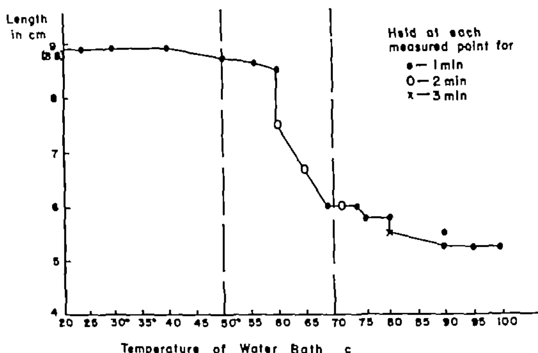


FIG. 10-8

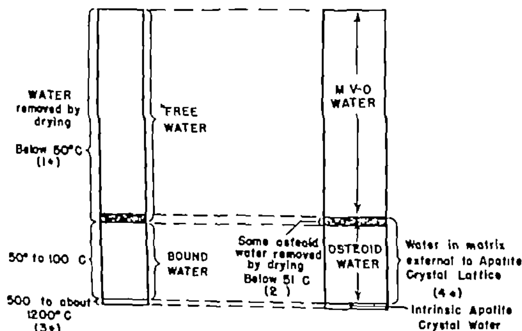


FIG. 10-9 1* Below shrinkage temperature of collagen. 2* Above shrinkage temperature of collagen. 3* Above the minimum melting temperature of the apatite crystals. 4 Osteoid water

Since some subsequent disintegration of puppy bones during EDTA decalcification occurs, even after this drying temperature, and since Amprino[34] found some matrix changes even in fully calcified matrix of dogs after drying at 38 C (although not nearly so marked as after 120°C drying) it appears that some water of constitution of the bone matrix is lost at drying temperatures of 50 C or below but the bulk of such calcified matrix water is lost by drying between 50 and 100°C

2* *Above Shrinkage Temperature of Collagen* After such drying temperatures even compact bone of adult dogs disintegrates during subsequent EDTA decalcification This disintegration was more marked in bone of very immature puppies than of adult dogs. It appears that more calcified *osteoid matrix* (or *bone matrix*) *water is present in the less completely* calcified bones of the puppies than of the dogs

3* *Above the Minimum Melting Temperature of the Apatite Crystals. The small bone crystals have been shown to grow in length, width, and* thickness when subjected to temperatures over 500°C[51]

4* *Osteoid Water* This is collagen fibril water and mucopolysaccharide water *in osteoid prior to osteoid calcification. During osteoid calcification,* this collagen and mucopolysaccharide water is reduced to a point where it may become practically synonymous with the "hydration shell" about the *periphery of apatite crystals An equilibrium apparently occurs at a certain* point in the calcification process at which the competition for osteoid water by the periphery of apatite crystals and their satellite ions, collagen fibrils, and mucopolysaccharide cement substance brings further displacement of water by inorganic matter to a dynamic equilibrium or end point. This end point represents the concept of "full calcification" in relation to osteoid water

Calcified Bone Matrix. Whereas the marrow once corrected for fat is considered to have a fairly constant density when dry and when hydrated, calcified osteoid, or in other words, calcified bone matrix, is considered to show a less constant density For instance it would appear that

1 There are species differences The density of "fully" calcified osteoid in man differs from that in the dog [34] or the turtle or the rat, or the rabbit.[21] This is probably a function of compactness of the collagen matrix which the bone cell of any particular type of animal may characteristically "weave." Rowland, Jowsey and Marshall [34] observed mineral density values in human bones and in dog bones. In human beings the hydroxyapatite per cubic centimeter of calcified bone matrix reached values around 1 37 Gm. whereas in the dog they achieved values of about 1.54 Gm In the rat the value rose as high as 1 7 Gm

2. A mixture of calcified osteoid and chondroid may occur If one is studying a bone specimen from rabbits one must be aware that, in the shafts of their long bones, islands of calcified chondroid from the epiphyseal mechanism may persist even after the animal has ceased to grow Calcified

epiphyseal cartilage in general is, when fully calcified, more dense than the bone with which it is associated[37] Robinson and Cameron[35] pointed out that the collagen fibrils in epiphyseal cartilage matrix occupy in human beings considerably less volume than collagen fibrils in bone matrix. As one can observe by electron microscopy the organic matter between the fibrils has per unit volume less density than the fibrils Thus, if crystals eventually fill most of the volume of epiphyseal cartilage and bone matrices not occupied by the dry organic components "fully" calcified epiphyseal cartilage matrix would be more dense than calcified bone matrix in the same animal species. However in dogs and in human beings, calcified cartilage is removed near the epiphyseal mechanism so that it does not appear in the shafts of the long bones intermixed with bone as in the rabbit. Therefore depending on the source of the bone this factor may play a part in the over all density of bone specimens.

As we consider calcifying matrix in bone, we must remember that, in enamel which has no cellular system mixed with its calcified matrix, water which could be removed from "fully calcified" enamel matrix is less than that which could be removed from incompletely calcified enamel matrix. Nevertheless, even when fully calcified, some water still could be removed from enamel specimens by drying at 100°C.

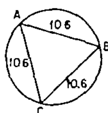
The subject of the water in bone matrix, where it is held and whether or not it is displaced by the inorganic material that collects in the organic matrix during calcification of bone matrix can be considered in this way

It has been noted further along in this chapter that osteoid A can apparently hold 114 84 per cent of its dry weight or 161 93 per cent of its dry volume as water while osteoid B can hold 128.5 per cent of its dry weight or 181 per cent of its dry volume as water

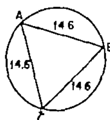
Rougvie and Bear[38] found in kangaroo tendon which has a densely packed collagen fibril matrix interspersed with cells, small blood vessels, and some perivascular tissue (similar in a very general way to uncalcified or decalcified bone matrix) that the dry collagen matrix considered as a unit would take up 120 to 127 per cent of its dry weight as water Not all this 120 to 127 per cent gain in weight was due to water taken up by the collagen fibrils since like bone the tendons contain mucopolysaccharides which are often more hydrophilic than collagen fibrils.[40] Furthermore the tendon samples were dried at 100 C and this, being above the shrinkage temperature of collagen, may have altered the collagen somewhat so that it would not fully rehydrate after such drying[39] However small angle x ray diffraction patterns were interpreted by Rougvie and Bear to show that, as the dried tendon samples came into equilibrium with pure water the average distance between the polypeptide chains in the fibril in the interband regions increased from 10 6 to 14 6 A During the hydration of the fibrils the macroperiods of the collagen fibrils in their long axis increased from 603 to 670 A in unrestrained specimens.

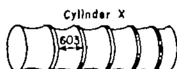
These data of Rougvie and Bear have been used by the author to calculate geometrically the average volumes of collagen fibrils in kangaroo tendon when hydrated and dry The difference represents a 110 8 per cent increase in volume during hydration The relevant calculations are

I

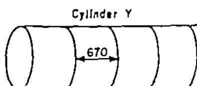


Hydration



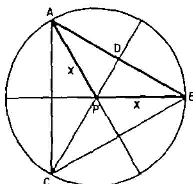


Hydration



Circle circumference $= 2\pi r$
Circle area $= \pi r^2$
Cylinder volume $= \pi r^2 h$

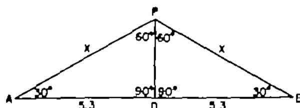
II



$AB = AC = BC = 10.6$ A

These lines outline a 60 60 60 triangle.

Bisecting angle ACB yields AD and DB both of which lines are then 5.3 A in length. Also angles PAD and $PBD = 30°$ each.



$X = AP$ or BP which are radii of the circle ABC

$$\text{The sine of angle } APD = \frac{AD \text{ side opposite}}{AP \text{ hypotenuse}}$$

$$\text{Therefore sine } 60 = \frac{5\ 3}{AP}$$

Natural sine of 60 $= 0\,8660 = \frac{5\,3}{AP}$

$$AP = \frac{5\,3}{0\,866}$$

$$AP = 6\,12\ \text{A}$$

III By the same method the radius of the circle $A\ B\ C' = 8\,43$ A

IV $\pi r^2 h =$ (for cylinder X) $3\,1416 \times (6\,12)^2 \times 603$ A
or volume of cylinder $X = 70\,953$ A^3
and volume of cylinder $Y = 149\,583$ A^3

Then

149 583
−70 953

78 630 A^3 = increase in volume between cylinder X and Y during hydration

$\frac{78\,630\ A^3 \times 100}{70\,953\ A^3} = 110\,8\%$ increase in volume of fibril during hydration

Since Bear[23] has recently demonstrated that the calculated density of dry collagen is about 1 41 and the water density is assumed to be 1 0 when incorporated in collagen fibrils

	Volume	×	Density	= Mass
Water	110 8	×	1 0	= 110 8
Collagen	100 0	×	1 41	= 141 0
Total	210 8	×	1 094	= 251 8

The hydrated collagen fibril has a density of about 1 1 The hydrated fibril is 53 per cent water and 47 per cent dry collagen by volume The fibril picked up about 80 per cent of its dry weight as water during hydration and so when hydrated is 44 per cent water and 56 per cent dry collagen by weight

It should be reemphasized that, as noted above the tendon matrix as a whole picked up about 120 per cent of its dry weight as water during hydration so that when hydrated it was about 60 per cent water and 40 per cent dry matrix by weight. It would appear that other elements of the whole tendon specimen besides collagen picked up water and that these other components were more hydroscopic than collagen per gram Nevertheless the collagen itself picked up the bulk of the water in the tendon specimens

In 1953 Nichols et al[41] published a paper entitled, "The Direct Measurement of the Extracellular Phase of Tissues" in which they analyzed



FIG. 10-10 Electron micrograph showing collagen matrix lying between two cells in a region of newly forming bone in the rat in the metaphyseal region just below the epiphysis The collagen fibrils are cut in almost exact cross section. In the small cut in the right upper corner of the figure one sees an enlargement showing crystals marked C" obliterating a fibril in one instance and lying between fibrils in another instance. At the mark of the arrow one sees that there are small calcific densities in the fibril. (*Reproduced by permission of J Biophys. Biochem Cytol.*, 3 1011–1016 1957)



FIG. 10-11 Electron micrograph of new bone formation in the metaphyseal area just under the epiphyseal line in the distal femur of a newborn infant An osteoblast is shown outside the calcified matrix along with other osteoblasts. Inside the calcified matrix is seen an osteocyte. The calcified matrix is very dense. There is a small area of newly formed osteoid which is not yet calcified lying between the calcified osteoid and the osteoblast. (Approximately ×6 000) (*Reproduced by permission of J Bone & Joint Surg.*, 40A 688 1958)

the achilles and patellar tendons from eight dogs They found that the water content was (on a fat-free basis) 667 Gm/kg Therefore the ratio of solids to water in these tendon specimens was about 1 2. In other words 100 Gm of such a collagen matrix commanded the presence of 200 Gm of water

If one calculates the amount of dry collagen in a bone specimen[12] and adds 80 per cent of that weight as water to permit "full" hydration of the bone collagen there is then more water in the specimen than one can find on analysis. This of course assumes that collagen of bone can hydrate to about the same degree as collagen of tendon. This assumption is supported

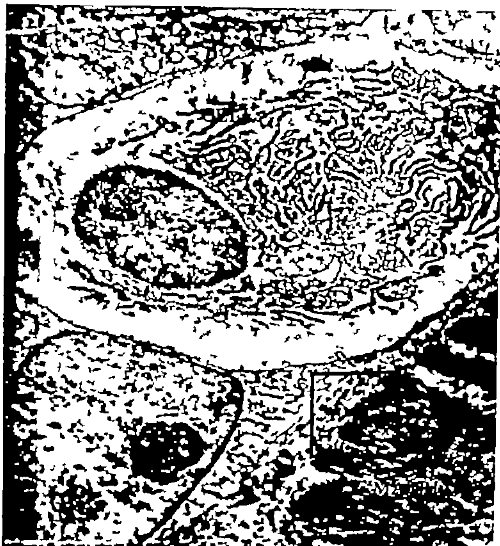


FIG. 10-12. Two osteoblasts are shown. The nucleus and the cytoplasmic reticulum are observed. *In each, the calcified bone matrix or calcified osteoid is seen* in the lower right-hand corner. A square is marked out including some of the cytoplasm of the osteoblast (its nucleus appears in the lower left-hand corner of the picture) and a strip of newly formed osteoid in which calcification is beginning. The square also includes some calcified osteoid matrix. This square is shown in more detail in Fig. 10-13. (Approximately ×12,000.)

by our own data[43] which show that after decalcification of bone matrix the amount of extra water taken up over and above that in the specimen prior to decalcification about equals the volume of inorganic matter lost and is about 110 per cent of the weight of the dried decalcified organic matrix. This is compatible with the concept, obtained by studying electron micrographs of bone, that the inorganic crystals in long-standing calcified osteoid not only surround but also permeate the fibrils (Fig. 10-10). Thus the concept has developed that the inorganic matter in bone matrix displaces

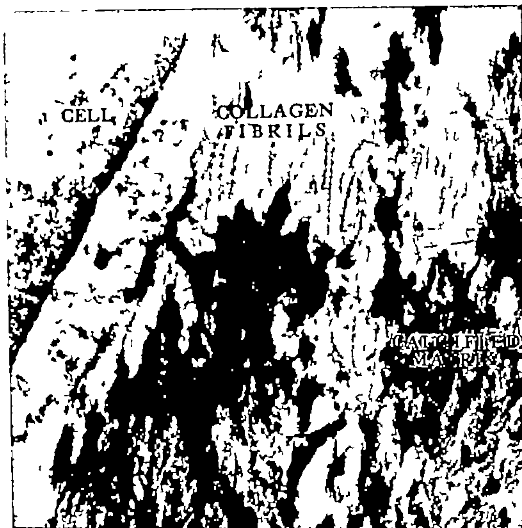


FIG. 10-13 Electron micrograph showing an area similar to that marked-out in the square in Fig. 10-12. The cell is seen in the left upper quadrant of the electron micrograph. The double-walled cell membrane is seen to separate the cell from the newly formed osteoid matrix. The collagen fibrils are well seen a short distance from the cell, and on and in these fibrils the calcification is proceeding. Also calcification occurs between the fibrils. (*Reproduced by permission of J Bone & Joint Surg* 40A, 694 1958)

not only the water in the cement substance but also the water in the collagen fibrils themselves up to the point where the osteoid water is bound by crystal surface phenomena as described by Neuman.[44]

It appears probable that a similarity of the calcification process in all five connective tissue matrices that normally calcify is the replacement of the bulk of matrix water by an almost equal volume of apatite crystals and their associated inorganic matter

In all these matrices electron microscopy shows that the cells produce the matrix in an uncalcified state As such it is probably hydrated like non calcifying matrices. The matrices of enamel, dentin epiphyseal cartilage

and bone appear to become relatively dry during the calcification process. However not all the water is normally displaced It would appear in fact that this bone matrix water is very important physiologically even though such water appears to be a small part of the total water in a normally calcified bone specimen.

Calcium phosphate crystals (hydroxyapatite) fill the osteoid[46] very soon after it is formed (Fig 10-11) This is not always true even in normal infants, but it is generally true [46] Sometimes in rapidly growing bone as noted by Park, "physiologic osteoid" can be seen in the light microscope and it is commonly observed in the most central lamellae of forming (as opposed to resorbing) osteons in the long bones The rapidity of calcification of osteoid after its formation has been appreciated in electron micrographs of newly forming metaphyseal bone from a newborn infant and in microradiographs of bone in which remodeling is occurring (Figs 10-12 to 10-14) Amprino and Engström[47] estimated that newly formed osteoid in normal animals quickly calcifies to about 70 to 80 per cent of "full calcification, full calcification being represented by the calcification of periosteal lamellae. After this level of calcification is reached in lamellae of secondary bone (Haversian osteons) there was comparatively slow completion of calcification to full calcification In periosteal lamellae calcification proceeded almost at once to full, or to as much calcification as possible, in the bone matrix characteristic of each animal studied. The average percentage calcification of Haversian systems in bones from several adult mammals lay between 92 and 96 per cent. Amprino and Engström[47] did not state how much of the total area of a bone section studied had less than full calcification, but the over-all calcification including primary and secondary bone more or less calcified would have had to average more than 92 per cent and less than 100 per cent of full calcification in the specimens they studied from several different animal species It could approach 100 per cent in bone specimens containing very few newly formed Haversian systems In a child, Amprino[19] found that the majority of Haversian units were about 80 to 85 per cent calcified, so that a lower *over-all* calcification, perhaps about 90 per cent, would be expected. In either event, average *over-all* matrix calcification approached 100 per cent of full calcification in bones from normal individuals of all ages.

Davies and Engström[48] found by interference microscopy that dry osteoid matrix had a fairly consistent density throughout decalcified bone sections regardless of the previous varying densities in different regions of the sections when the bone was undecalcified

The uniform density of the dry organic matrix of bone at least after the initial process of calcification, implies that the amount of cement substance and collagen solids per unit volume does not change significantly at least after initial calcification (i e to about 70 per cent full calcification) has been achieved Of course there may be variations of the collagen-mineral

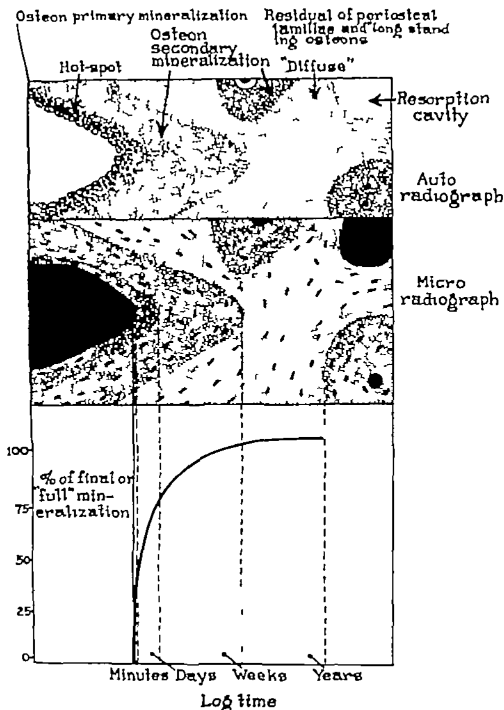


FIG. 10-14 A schematic drawing showing the relationships of those features seen in autoradiographs, microradiographs of bone and a roughly estimated graph to illustrate the time relationships associated with these things seen in the autoradiographs and microradiographs. (*Drawing based on a sketch presented to the author by Jenifer Jowsey* 1958.)

ratio between lamellae in one osteon[34] or in different osteons which will make some regions of bone appear less calcified than others in microradiography and yet when mineral fills all the available water space these areas of slightly different density as determined by microradiographs will be calcified as fully as possible.

On the other hand, it is known that both mucopolysaccharides and collagen fibrils can retain large amounts of water In a nonexpanding organic matrix of constant density such as osteoid, something must be lost to permit the ingress of inorganic space filling matter during calcification. It would appear that the substance displaced from bone matrix during its calcification is water

The next point to consider is whether *all* the water in freshly formed uncalcified osteoid is apt to be displaced during calcification.

Calcium strontium, sodium and many other charged ions require water for relatively rapid movement at body temperature This water must have a certain degree of freedom so that it can hydrate the ions during their movement from one place to another Ions of calcium move through fully calcified or almost fully calcified bone matrix very slowly whereas such ions can move through incompletely calcified bone matrices very rapidly

Tomlin, Henry and Kon in 1953[49] reported experiments in which young rats were kept on a diet containing a constant quantity of radioactive calcium for a long period These young rats developed a rather constant blood serum specific activity calcium[45] radioactive/calcium nonradioactive = specific activity Even in young rats with a constant specific activity of the serum, the bone already formed at the beginning of the experiment equilibrated slowly with the blood serum. During their relatively long-term experiments the specific activity of the long-established bone reached only about 23 per cent in 100 days and that activity remained fairly constant thereafter On the other hand, newly forming bone (bone formed during the course of the experiment) had a specific activity of about 80 to 100 per cent.

In 1955 Tomlin Henry and Kon[50] again reported experiments on rats kept on a radioactive calcium diet for long periods. Again the equilibration was slow between the serum calcium and the bone calcium in regions of long-standing bone but rapid in regions of newly forming bone As before, the specific activity in newly forming regions approximated that of the blood serum, whereas in the long-standing bone even after equilibration was reached the specific activity was only about 25 per cent of the serum They concluded that there was an exchangeable fraction of calcium atoms in long standing bone of the rat of not more than 0.2 per cent on any one day and about 1 per cent or less per week

As for the finding that the total uptake of the old rat bone is limited by some factor to about 25 per cent of its calcium atoms, it should be noted that crystals of about the size observed in the third stage of calcification[45]

have 20 to 30 per cent of their calcium and phosphorus atoms in the outermost molecular layer of such crystals[22] Possibly this surface measurement is related to the figure of about 25 per cent obtained by Henry Tomlin and Kon. In other words the percentage of molecules on the surface of crystals established at the time the experiments were started may be a practical limiting factor for the *amount* of ion exchange in areas of long-standing bone at least for the period covered by the experiments of Tomlin Henry and Kon It is of interest that the exchangeable PO_4 fraction of such crystals still associated with their matrix but dispersed in a water bath showed 25 to 30 per cent exchangeable phosphate[31]

About 80 to 100 per cent specific activity could be *quickly* achieved in the bone forming during the experiment. The crystals in newly forming bone might be expected to have both radioactive and nonradioactive ions built throughout them rather than just on their surfaces Such crystals forming while the serum specific activity was constantly elevated would therefore be expected to have a specific activity approximately the same as that of the blood serum

The *rate* of calcium ion exchange in fully calcified bone matrix could conceivably be slow for two reasons (1) the pathway between the blood and the crystal surfaces through the calcified osteoid could be obstructed in nearly fully calcified bone or (2) there might be no place for the ions to go in the fully calcified bone The latter point does not seem to be reasonable because there are plenty of crystal surfaces in both fully and incompletely calcified bone The crystals do not appear to us to form a continuous lattice throughout the calcified matrix, as suggested by some investigators The crystals do not change sufficiently in size (i e., average size) as far as Robinson and Watson could determine[22] to make any significant difference in the rate of equilibration between the calcium on the crystal surfaces in newly formed and long-standing bone and the blood serum calcium. In support of this point, Amprino found that if he removed the organic matter from bone leaving the crystals[32] the *differences* in speed of exchange as well as the total amount of exchange between newly formed and long-established bone regions became considerably reduced or disappeared altogether Such an experiment being performed in vitro would appear to reflect ion surface exchange of crystals already present and would not necessarily represent any crystal accretion

It appears that the big difference in the *rate* of exchange found in vivo between newly formed and long-established bone must be due to an interference with ion migration through the organic matrix of long-established bone and not due to lack of crystal surface sites for ion exchange or differences in surface area of crystals in newly formed and established bone The following mechanism for the blocking of ion migration in calcified organic matrices has been suggested.[33] Any water remaining in the fully calcified osteoid matrix is so completely immobilized that it transports inorganic ions

that need water for transport very slowly through the matrix The pores or pathways between solids in fully calcified matrix may become so small that in a short time interval almost no ion migration may occur through them, particularly if the ion is charged since the pore walls also carry a charge

If the water content of decalcified bone is any guide it would appear that prior to calcification osteoid can hold a relatively large amount of water From data obtained in our laboratory on decalcified bones it can be calculated that the weight of water which decalcified osteoid can hold is in excess of the dry weight of the osteoid This is comparable to Rougvie and Bear's finding on rehydration of another collagenous matrix, kangaroo tail tendon,[39] wherein the matrix could hold a little over 120 per cent of its dry weight in water when fully hydrated

It is proposed that there are two major water compartments in bone (1) the marrow vascular-osteocyte space water or water outside the bone matrix and (2) water in the bone matrix or osteoid.

It is proposed that the *water in osteoid* is divisible into two compartments

One water compartment in osteoid consists of water that is "free" enough to hydrate inorganic ions and thus participate in their movement through the osteoid Such water is believed to be present for the most part in osteoid that is not completely calcified and might be called "free water of potentially calcifiable osteoid space" There would be a relatively small amount of this water in fully calcified bone matrix. Some of this water may be removed by drying at 50 C.

The other water compartment consists of water that is so firmly "bound that it is of no use in the movement of ions through the calcifying osteoid over short time periods This compartment in turn probably consists of two parts

1 *Water of Constitution* Drying a bone specimen by heating at 100 C or by prolonged and strenuous freeze-drying methods removes about 3 to 4 per cent more of the original weight than drying at 50°C in air or by desiccation in a vacuum over silica gel but it also makes the organic matrix more soluble so that during subsequent decalcification there is a large loss of the matrix Therefore it is suggested that drying at this higher temperature removes water which somehow formed an integral part of the organic matrix itself For instance prior to its removal it may have been associated with the hydrogen bonds in collagen fibrils between a hydroxyl group of a prosthetic group on one protofibril and the imide group on another protofibril [44] and can be removed when the shrinkage temperature of collagen is exceeded. The water of crystallization of apatite is also considered to be part of this water of constitution Previously it seems that this water of inorganic crystallization was considered to be all the water of constitution in bone It is said to comprise a very small part of the weight of the crystals[42,44] and therefore is a very small part of bone water We

suggest that it is only part of the water of constitution the other part belonging to the collagen fibrils and possibly also to the mucopolysaccharides. Actually the water of constitution of hydroxyapatite crystals is not significantly reduced until temperatures of about 600°C are reached. Water removed by drying methods which exceed the shrinkage temperature of mammalian collagen (i e over 60 C)[16] is probably so firmly bound (mostly to organic but possibly some to inorganic structures) in living bone that it can only slowly take part in any movement of inorganic ions through osteoid or crystals in vivo

2 *Bound Water of the Potentially Calcifiable Osteoid Space* The other anatomic division of "bound" osteoid water in fully or nearly fully calcified bone is postulated as being that water which, although part of the "free osteoid water" at the beginning of calcification became immobile during the calcification process As calcification proceeds in a fixed volume of bone matrix the space for water between the bone crystals and other solids in the osteoid must be reduced If one considers the osteoid matrix as a structure in which the solids consist of apatite crystals and associated inorganic ions, collagen fibrils, collagen molecules not incorporated into fibrils and mucopolysaccharide molecules, then when calcification approaches completion the spaces between these various solid components must become extremely small to the point where water molecules and hydrated ions can no longer move between them Thus a situation obtains which appears to be similar to that described by Sollner for plastic porous membranes[37] wherein the size of "pores" (in the extracellular osteoid in the case of calcified bone matrix) is extremely small while the density of polar charges around the borders of the pores is so high that movement of hydrated ions becomes restricted or almost impossible Thus a terminal period is probably reached in calcifying bone matrix when ion exchange becomes first obstructed and then progressively slowed to a point where it will be almost nonexistent, at least in short-term isotope experiments. Water of the potentially calcifiable osteoid space remaining in the osteoid when calcification is full or as complete as possible would represent the irreducible minimum of the hydration layer about the bone crystals and might reasonably be called the "bound water of the potentially calcifiable osteoid space." Neuman and Neuman[33] have discussed a "hydration layer" about the crystal in some detail It seems to us that in fully calcified bone such a hydration layer represents the "bound" remains of the "free" water which hydrated the osteoid before it was fully calcified Ions can interact with the crystal surfaces of bone only by passing through the matrix and the "hydration shell" of the crystals. The ease with which an ion can get through depends on its size and charge Calcium and sodium which are heavily hydrated have a large effective size Phosphate on the other hand, although it is a multiatomic ion is not hydrated, and its effective size is, therefore much smaller than one might otherwise expect. Hydrogen ion

potassium ion and chloride ion would all diffuse more easily than phosphate, but it is possible that phosphate could diffuse more readily than calcium. Of course a noble gas such as radon with no charge or hydration shell would move through spaces impassible to calcium Radon is said "to escape from the bone crystals by virtue of its recoil energy derived from the disintegration of radium." [83] This force apparently contributes to its ability to move through fully calcified bone matrix.

Therefore, although the bulk of the water is apparently in the marrow-vascular-osteocyte space in a bone any small amount of *mobile* water in

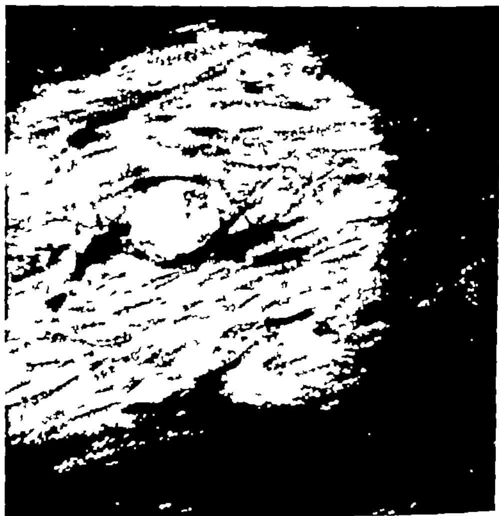


Fig. 10-15 Electron micrograph of a section of partly decalcified bone matrix from the outer cortex of the adult human rib The canaliculi is seen in the center The decalcifying solution extended out from this canaliculi into the calcified osteoid exposing the collagen fibrils which were filled with and covered by crystals prior to decalcification. Note that the matrix is practically all decalcified or not decalcified at all along a sharply demarcated zone about 500 A in width. (About ×35 000) (*Reproduced by permission of Anat Rec* 114 409 1952.)

bone matrix would be of extreme physiologic significance The free oste
water would, according to the concept just stated be mostly located
incompletely calcified osteoid where ion migration from blood to b
crystals is more rapid. Even in fully calcified bone there is a little oste
water but it is bound so that ion transit is very slow Small as it may
in relation to all the water in a bone specimen the osteoid water of
tentially calcifiable osteoid space appears to form the final and critical p
of the pathway for ion exchange between the hydroxyapatite crystals
the bone matrix and the blood.

It appears to us that 100 C drying of bone removes "water of const
tion" of the collagen but not of the bone crystals. Such drying may r
remove the bound water of potentially calcifiable osteoid space

There is one observation based upon electron microscopy of bone s
tions which in my opinion demonstrates that fully or nearly fully calci
bone matrix is practically dehydrated A piece of bone from the subp
osteal lamellae of the outer cortex of the rib of an adult human male v
placed in an EDTA solution while still fresh It was removed from
EDTA solution before complete decalcification had occurred, wash
fixed in osmium tetroxide embedded in methacrylate and sectioned v
a glass knife (Fig 10-15)

The sections showed that the decalcifying solution had penetrated
lacunae and canaliculi. From these it had spread centrifugally through
bone matrix. The line between calcified matrix and completely decalci
matrix is sharp Only over a distance of about 500 to 1 000 A can one
partial crystal dissolution If the versene could pass freely through
matrix, a different picture would be seen Partial dissolution of crystals o
a wide area would be seen There might be *more* complete dissolution
the regions immediately surrounding a lacuna and its canaliculi and i
complete dissolution at a distance but it seems unreasonable to me t
there would be a *sharp* zone of division, only about 500 to 1 000 A
width between complete decalcification and no apparent decalcificat
if the aqueous versene solution could permeate the whole section at or
It appears to me that it can permeate only matrix from which it has l
removed the crystals, and so the pattern in these electron micrograph
produced which shows removal of crystals only at the decalcified-calci
matrix interface

STANDARD OSTEOID PATTERNS It will be noted in Fig. 10-4 that
majority of data points of 488 bone samples having a density of 2 05
1 85 when hydrated lie between 2 36 and 2.26 on the dry density sc
Specimens falling in this range are subject to fewer laboratory errors v
the techniques we use for determining specific gravity and density [21] Th
bone specimens are comparatively large making the figures obtained by
weighing methods on them more significant In general the reproducibi
of analyses of such bone samples from the tibial cortex of mature heal

dogs is remarkable Since bone specimens lying in this region of Fig 10-3 appear to be more uniform than elsewhere, standard patterns of analysis of the marrow vascular-osteocyte space and of the calcified osteoid have been based on analysis data of such specimens.

The first *standard A osteoid* is based on the average density figure in Table 10-2 for bone having the characteristics D^h2.05 and D^d2.3242. As noted above these figures are somewhat modified by the data from all 228 bone specimens involved in the derivation of the figures in Table 10-2.

Table 10-4

Density of hydrated bone specimen	*Difference between weight loss of specimen when dried at 50°C and 100°C grams per 100 cc of hydrated specimen's weight*	*Grams per 100 cc* *Weight loss (water) at 100°C drying*	*Weight loss (water) at 50°C drying*
2.05	3 40	10 1	6.7
2.00	3 44	12.2	8.76
1 95	3 48	14.45	10.9?
1.90	3.52	16.?	13.18
1.85	3.57	19.2	15.63

The second *standard B osteoid* is based on bone having the charac teristics D^h1 95 and D^d2.35 obtained from an average of our previously published figures[21] for 92 dog bone specimens having a density of 2.04 to 1 85 when hydrated and a density of 2 35 when dried As can be seen in *Table 10-4 bones having a hydrated density of 2.05 lose 3 4 per cent* of their hydrated weight on an average when dried at 100°C after they have been previously dried at 50 C and in the case of bones having a hydrated density of *1 95 the specimens lose* on an average of *3 48* per cent of their original hydrated weight when heated between 50 and 100 C. Therefore the amount of standard osteoid was based on these figures. The weight loss at 50 C drying was used as the average amount of water lost from the marrow vascular-osteocyte space and on such figures the amount of standard marrow was based. For instance at D^h2.05 and $D^{d(h)}$2.3242, the marrow associated with standard osteoid contained 6 7 Gm per 100 cc water whereas at D^h1 95 and $D^{d(h)}$2.35 the marrow associated with standard osteoid contained 11 7 Gm per 100 cc water on the basis of total specimen weight. The over all weight and volume of the marrow-vascular-osteocyte space could then be derived from the marrow vascular-osteocyte water weight (or volume) on the basis of the standard mar row pattern in Table 10-3 for each of these specimens of bone The weight of each bone specimen (100 Gm) minus the weight per cent of the marrow vascular-osteocyte space and osteoid water gave the weight

per cent of the dry matter in the calcified bone matrix This in turn was divided between its inorganic and organic fractions by application of their respective densities, namely 3 and 1 41 In this way patterns of bone specimens in which standard osteoid A was combined with standard marrow and standard osteoid B was combined with standard marrow were derived. These patterns follow

Table 10-5

STANDARD OSTEOID A AND STANDARD MARROW

Components	*Mass %*	*Density*	*Volume*	*Volume %*	*Grams per 100 cm³*
M V-O					
Water	0.70	1 00	6 70	13 74	13 74
Inorganic	0 05	3 00	0 02	0 03	0 09
Organic	0.87	1 41	0.62	1.26	1 78
Total	7 62	1 04	7.34	15 03	15 61
Osteoid					
Water	3 40	1 00	3 40	6.97	6.97
Inorganic	66 67	3 00	22.22	45.50	136.68
Organic	22.31	1 41	15.82	32.44	45 74
Total	92.38	2.23	41 44	84.07	180 30
Total specimen	100 00	2.05	48 78	100 00	205 00

M V-O $\quad D^{A(a)} = \frac{0\ 9137}{7.3318} = 0\ 12$

$D^{A(b)} = \frac{0\ 9137}{0\ 6309} = 1\ 45$ $\qquad D^{A} = 1.04$

Osteoid $\quad D^{A(a)} = \frac{88\ 9854}{41\ 44507} = 2.15$

$D^{A(b)} = \frac{88.9854}{38.01567} = 2.34$ $\qquad D^{A} = 2.23$

Total $\quad D^{A(a)} = \frac{89.8991}{48\ 78047} = 1.84$

$D^{A(b)} = \frac{89.8991}{38\ 67951} = 2.32$ $\qquad D^{A} = 2.05$

VARIATIONS IN STANDARD OSTEOID A ANALYSIS PATTERN WITH VARIATIONS IN DEGREE OF ITS CALCIFICATION Since the calcified osteoid contributes 92 3854 per cent of the mass of the analysis pattern of a bone specimen composed of standard marrow plus standard osteoid A if one multiplies the weight percentages by 1 082422 one can construct a standard osteoid pattern comparable to the standard marrow pattern.

Table 10-6

STANDARD OSTEOID A PATTERN AND VARIATIONS

Components	*Basis of grams per 100 Gm*			*Basis of cm³ per 100 cm³*		
	Mass %	*Density*	*Volume*	*Volume*	*Density*	*Volume %*
Water	3.08	1.0	3.08	3.08	1.0	8.20
Inorganic	72.17	3.0	24.06	24.06	3.0	63.62
Organic	24.15	1.41	17.13	17.13	1.41	38.18
Total	100.00	2.23	44.87	44.87	2.23	100.00

When bone matrix first forms, it is not calcified (Figs 10-11 to 10-13) *However* calcium and phosphate crystals soon appear in it unless the animal is rachitic or unless the bone matrix is abnormal as in certain osteogenic sarcomas (Fig 10-16) In this figure it will be seen that the epiphyseal cartilage fibrils are very much smaller than the collagen fibrils in the bone matrix and, in general, are less closely packed whereas the bone matrix fibrils are larger periodicity is well seen in this osmic acid stained preparation, and the fibrils are more closely packed than those in the epiphyseal cartilage It is obvious that the collagen fibrils in bone per unit volume (since these sections are of equal thickness) contribute a greater mass than the collagen fibrils in the epiphyseal cartilage per unit volume. *Gradually the matrix becomes calcified (Fig 10-14) Full calcification* simply means for a bone matrix of any one species of animal that it holds as much inorganic matter as possible under normal conditions It is inconceivable that all the water in a matrix even at full calcification is displaced by mineral since Ca ions can slowly permeate such bone,[11, 58] and they need water for transport.

"Theoretically complete calcification" and full calcification" are therefore different since by the former one means that all the water has been displaced by mineral whereas the latter means that as much matrix water as can normally be displaced by mineral has been so displaced. Not knowing exactly how to measure full calcification we have related the calcification percentages to complete theoretical calcification in this work. This will undoubtedly cause some confusion, for the custom has been to relate all differing degrees of calcification to full calcification which is found in each sample of bone in areas where there are long-established osteons or periosteal lamellae Complete theoretical calcification is found in our material by calculating the situation which would exist if all the water in osteoid were replaced by mineral This has at least one advantage. It permits one to say that full calcification is always less than complete theoretical calcification, thus implying that even at full calcification some water space exists in the osteoid In our present thinking, theoretically complete calcification

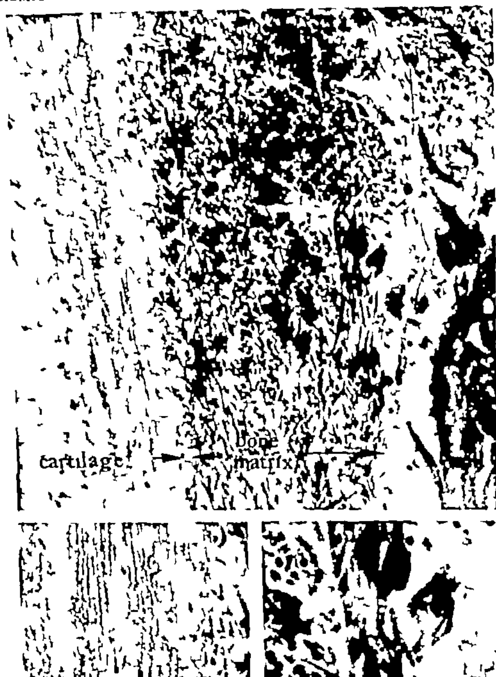


FIG 10-16 Section from the metaphyseal region of a rat in which florid low phosphorus rickets was present *Upper* At the lower right hand corner one sees a part of a cell—an osteocyte. Next to this in the left hand side of the picture is seen the collagen matrix, and on the left half of the picture is seen the cartilage matrix. This section was not decalcified but was obtained at the point where osteoid was being laid down on uncalcified cartilage matrix. *Lower right* An enlargement of part of the bone matrix *Lower left* An enlargement of part of the cartilage matrix.

Table 10-6

STANDARD OSTEOID A PATTERN AND VARIATIONS

Components	Basis of grams per 100 Gm			Basis of cm^3 per 100 cm^3		
	Mass %	Density	Volume	Volume	Density	Volume %
Water	3.08	1.0	3.68	3.08	1.0	8.20
Inorganic	72.17	3.0	24.06	24.00	3.0	53.62
Organic	24.15	1.41	17.13	17.13	1.41	38.18
Total	100.00	2.23	41.87	44.87	2.23	100.00

When bone matrix first forms it is not calcified (Figs. 10-11 to 10-13) However calcium and phosphate crystals soon appear in it unless the animal is rachitic or unless the bone matrix is abnormal as in certain osteogenic sarcomas (Fig 10-16) In this figure it will be seen that the epiphyseal cartilage fibrils are very much smaller than the collagen fibrils in the bone matrix and, in general are less closely packed whereas the bone matrix fibrils are larger periodicity is well seen in this osmic acid stained preparation and the fibrils are more closely packed than those in the epiphyseal cartilage It is obvious that the collagen fibrils in bone per unit volume (since these sections are of equal thickness) contribute a greater mass than the collagen fibrils in the epiphyseal cartilage per unit volume. Gradually the matrix becomes calcified (Fig 10-14) Full calcification simply means for a bone matrix of any one species of animal that it holds as much inorganic matter as possible under normal conditions. It is inconceivable that all the water in a matrix even at full calcification is displaced by mineral since Ca ions can slowly permeate such bone,[49, 50] and they need water for transport.

Theoretically complete calcification" and "full calcification" are therefore different since by the former one means that all the water has been displaced by mineral whereas the latter means that as much matrix water as can normally be displaced by mineral has been so displaced Not knowing exactly how to measure full calcification, we have related the calcification percentages to complete theoretical calcification in this work. This will undoubtedly cause some confusion for the custom has been to relate all differing degrees of calcification to full calcification which is found in each sample of bone in areas where there are long-established osteons or periosteal lamellae Complete theoretical calcification is found in our material by calculating the situation which would exist if all the water in osteoid were replaced by mineral. This has at least one advantage. It permits one to say that full calcification is always less than complete theoretical calcification thus implying that even at full calcification some water space exists in the osteoid In our present thinking, theoretically complete calcification

4 $\frac{58.2402}{24.1514} = 2.41171$ — 8.3210 water; 58.2402 inorganic; 24.1514 organic; 90.7186 Gm

$\frac{2.41171}{3.4453} = 70\%$

5 $\frac{49.0253}{24.1514} = 2.00718$ — 11.0046 water; 49.0253 inorganic; 24.1514 organic; 85.1713 Gm

$\frac{2.00718}{3.4453} = 60\%$

6 $\frac{41.6045}{24.1514} = 1.72265$ — 13.8682 water; 41.6045 inorganic; 24.1514 organic; 79.0241 Gm

$\frac{1.72265}{3.4453} = 50\%$

Osteoid A characteristics

1 100% calcification $D^{(a)}\ \frac{107.3604}{44.8650} = 2.4$

2. Standard (86.73% calcification) $D^{A}\ \frac{100\ \text{Gm}}{44.8650\ \text{cm}^3} = 2.23$

$D^{A(w)}\ \frac{96.3198\ \text{Gm}}{44.8650\ \text{cm}^3} = 2.15$

$D^{A(a)}\ \frac{96.3198\ \text{Gm}}{41.1848\ \text{cm}^3} = 2.34$

3 80% calcification $D^{A}\ \frac{96.2659}{44.8650} = 2.15$

$D^{A(w)}\ \frac{90.7185}{44.8650} = 2.02$

$D^{A(a)}\ \frac{90.7185}{39.3176} = 2.30$

4. 70% calcification $D^{A}\ \frac{90.7186}{44.8650} = 2.02$

$D^{A(\ )}\ \frac{82.3976}{44.8650} = 1.84$

$D^{A(a)}\ \frac{82.3976}{36.5440} = 2.25$

5 60% calcification $D^{A}\ \frac{85.1713}{44.8650} = 1.90$

$D^{A(w)}\ \frac{74.0767}{44.8650} = 1.65$

$D^{A(a)}\ \frac{74.0767}{33.7704} = 2.10$

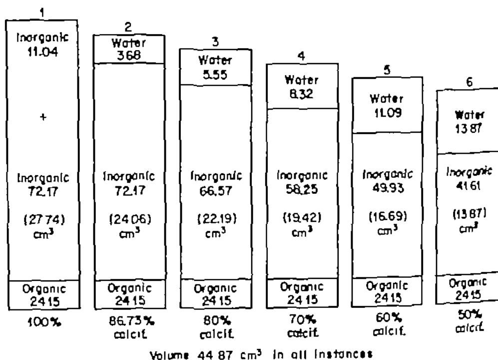
6. 50% calcification $D^{A}\ \frac{79.0241}{44.8650} = 1.7$

$D^{A}\ \frac{65.7559}{44.8650} = 1.47$

$D^{A}\ \frac{65.7559}{30.0905} = 2.12$

may never exist or if it does it is rare possibly in an old sequestrum in an osteomyelitic abscess or in bone the cells of which have died in situ.

If we are correct in assuming (1) that the water removed between 50 and 100 C drying comes from osteoid and (2) that potentially the crystals could fill all this water space then 100 per cent calcification would mean that the last remaining space in the osteoid occupied by water is replaced by an equal volume of inorganic matter having a density of 3



Proportions of water inorganic and organic matter in variations of osteoid A

100% calcification

1 Inorganic 83.2090 — Organic 24 1514 = 100% calcified

$\frac{83.2090}{24\ 1514}$ = 3 4453 = 100% calcified

11.0406 water
72.1684 inorganic
24 1514 organic
107.3604 Gm

2 Standard

$\frac{72\ 1685 \text{ inorganic}}{24\ 1514 \text{ organic}}$ = 2.9882

$\frac{2\ 9882}{3\ 4453}$ = 86.73% calcified

3.680 water
72.1685 inorganic
24 1514 organic
100 00 Gm

3 $\frac{60.5671}{24\ 1514}$ = 2.75624

$\frac{75624}{3\ 4453}$ = 80%

5.5474 water
60.5671 inorganic
24 1514 organic
96.2659 Gm

at least in the canine the bone matrix is similar in puppies and dogs inas much as the same upper limit of inorganic/organic ratio per unit volume exists in both. This suggests a species specificity The organic density remains about the same regardless of calcification percentage according to Davies and Engström

Thus we do not for the present hesitate to use data obtained from bone samples of adult dogs for derivation of full" (86 73 per cent) calcification and the more theoretic "100 per cent" calcification figures

Table 10-7

STANDARD OSTEOID B AND STANDARD MARROW

Components	*Mass %*	*Density*	*Volume*	*Volume %*	*Grams per 100 cm³*
M V-O					
Water	11 71	1	11 71	22.81	22.84
Inorganic	0 08	3	0 03	0 05	0 16
Organic	1.52	1 41	1 08	2.10	2 00
Total	13.31	1 0382	12.82	24 99	25.06
Osteoid					
Water	3 48	1	3 48	6 70	6 70
Inorganic	63 92	3	21 31	41.55	124 05
Organic	19 28	1 41	13 67	26 07	37 00
Total osteoid	86.68	2.25	38.47	75 01	169 04
Whole bone specimen	100 00	1 95	51.28	100 00	195 00

Then the density of the osteoid when hydrated is 2 2531 the density of the marrow vascular-osteocyte space when hydrated is 1 0382 and the density of the whole bone specimen when hydrated is 1 95

M V-O $$D^{(a)} \frac{1.5975}{12.8176} = 0\ 125$$

$$D^{(s)} \frac{1.5975}{1\ 1020} = 1\ 45$$

Osteoid $$D^{(a)} \frac{83.2078}{38\ 4645} = 2.16$$

$$D^{(s)} \frac{83.2078}{34.0845} = 2.38$$

Total $$D^{(a)} \frac{81.8053}{51.2821} = 1\ 65$$

$$D^{(s)} \frac{84.8053}{36\ 0874} = 2\ 35$$

In each of these variations on the standard osteoid A pattern, the number of grams of organic matter having a density of 1 41 when dry is the same in relation to the volume upon which these variations are based. The same amount of organic matter holds an equal volume composed of water and inorganic matter the relative volumes of water and inorganic matter having a reciprocal relationship In other words 24 15 Gm of organic matter has a volume of 17 12 cm^3 This amount of organic matter can hold 27 74 cm^3 of either water or inorganic matter or a mixture of water and inorganic matter the volume of which equals 27 74 cm^3 This implies that before calcification of 24 15 Gm of osteoid it can hold 27 74 Gm of water or 114 87 per cent of its dry weight as water or 27 74 cm^3 water/17 13 cm^3 organic = 161 93 per cent of its dry volume as water

This organic figure is based on analysis of bone samples of the density range 2 05 to 1 90 (D^A) in which laboratory errors are least and in which fairly uniform results are obtained with multiple specimens There are however variations in the analyses of bone samples from puppies which may be largely due to method errors. One must realize that the organic matrix might be different in puppy bone in this way The organic matrix might hold more water before calcification per gram or per cubic centimeter of organic matter thus permitting more inorganic replacement of the water in it when in time it became fully calcified

There appears to be some species differences[21, 34] so that the calcified bone matrix of turtles and rabbits is more dense than that of dogs while calcified human osteoid appears less dense than that of dogs.

Robinson and Watson concluded that the fibril diameter and packing density was less in the subperiosteal bone of the infant rib than in the adult rib [23]

Epiphyseal cartilage has a fibril diameter that is much smaller than that of bone and the fibrils are loosely packed compared with those in bone.[38] This situation appears to permit more inorganic matter relative to organic matter in fully calcified epiphyseal cartilage and as noted by Owen Jowsey and Vaughn[37] the density of fully calcified cartilage (as determined by microradiography) is greater than even the most fully calcified bone in the rabbit

Therefore further investigations may show that the osteoid of a very immature member of any one animal species can hold more water before calcification and more inorganic matter after calcification per unit of weight or volume than can the osteoid of a mature member of the same species. However for the present, direct experimental evidence seems to contradict this possibility Rowland Jowsey and Marshall [34] noted that whereas there was more variation in the density of calcified osteoid in young than in older dogs there were no areas of absolutely greater density in the bones of young dogs than in the bones of older dogs. Thus it would appear that

Osteoid B density ratios.

1. 100% calcification

$$D^{A(b)}\ \frac{108.0288}{44.3712\ \text{vol}} = \frac{108.0288}{15.7767 + 28.5945} = 2.43$$

2. Standard (85.96% calcification)

$$D^{A}\ \frac{100}{44.3712} = 2.25$$

$$D^{A(a)}\ \frac{95.9856}{44.3712} = 2.16$$

$$D^{A(b)}\ \frac{95.9856}{40.3568\ \text{vol}} = \frac{95.9856}{44.3712 - 4.0144} = 2.38$$

3. 80% calcification

$$D^{A}\ \frac{96.5907}{44.3712} = 2.18$$

$$D^{A(a)}\ \frac{90.8716}{44.3712} = 2.05$$

$$D^{A(b)}\ \frac{90.8716}{38.6531} = 2.35$$

4. 70% calcification

$$D^{A}\ \frac{90.8722}{44.3712} = 2.05$$

$$D^{A(a)}\ \frac{82.2039}{44.3712} = 1.85$$

$$D^{A(b)}\ \frac{82.2039}{35.7929} = 2.30$$

5. 60% calcification

$$D^{A}\ \frac{85.1537}{44.3712} = 1.92$$

$$D^{A(a)}\ \frac{73.7161}{44.3712} = 1.66$$

$$D^{A(b)}\ \frac{73.7161}{32.9336} = 2.24$$

6. 50% calcification

$$D^{A}\ \frac{79.4352}{44.3712} = 1.79$$

$$D^{A(a)}\ \frac{65.1384}{44.3712} = 1.47$$

$$D^{A(b)}\ \frac{65.1384}{30.0744} = 2.17$$

Thus, 22.25 Gm organic of D^A 1.41 has a volume of 15.78 cm^3. Theoretically this amount of organic can hold 28.59 cm^3 of water or inorganic matter. This implies that prior to calcification of 22.25 Gm of organic matter osteoid B can hold 28.59 Gm of water.

$$\frac{28.5945\ \text{Gm water}}{22.2452\ \text{Gm organic}} = 128.5\%$$

Table 10-8

STANDARD OSTEOID B PATTERN AND VARIATIONS

Components	Basis of grams per 100 Gm			Basis of cm³ per 100 cm³		
	Mass %	Density	Volume	Volume	Density	Volume %
Water	4 01	1 0	4 01	4.01	1	9 05
Inorganic	73 74	3 0	24.58	24.58	3	55.40
Organic	22 25	1 41	15 78	15 78	1 41	35.50
Total osteoid	100 00	2.25	44 37	44.37	2.25	100.01

Proportions of water inorganic and organic matter in variations of osteoid B

1 *100% calcification.* The standard osteoid pattern has 73 74 Gm inorganic to 22.25 Gm organic However if the 4.01 cm³ of water were replaced by inorganic of density 3 so that 100 per cent calcification was theoretically achieved, then there would be 73 74 Gm + 12.04 = 85 78 Gm inorganic to 22.25 Gm of organic *thus* the ratio 3.86 = 100% calcification

Analysis	
0.0	water
85 7836	inorganic
22.2452	organic
108 0288	Gm osteoid

2 The standard, however has a ratio of 73 74/22.25 = 3.31 Therefore the percentage calcification of the standard osteoid is 3.31/3.86, or 85.96%.

4.0144	water
73 7404	inorganic
22.2452	organic
100.0000	Gm osteoid (hydrated)

3 80% calcification

$$\frac{68.6264}{22.2452} = 3.09$$

$$\frac{3\ 0850}{3.8563} = 80\%$$

5 7191	water
68 6264	inorganic
22.2452	organic
96.5907	Gm

4 70% calcification

$$\frac{60\ 0487}{22.2452} = 2\ 70$$

$$\frac{2.6994}{3.8563} = 70\%$$

8.5783
60.0487
22.2452
90.8722

5 60% calcification

$$\frac{51\ 4709}{22.2452} = 2.31$$

$$\frac{2.3138}{3.8563} = 60\%$$

11 4370
51 4709
22.2452
85.1537

6 50% calcification

$$\frac{42.8032}{22\ 2452} = 1\ 9282$$

$$\frac{1\ 9282}{3\ 8563} = 50\%$$

14.2968
42.8032
22.2452
79 4352

collagen packing and size may be very similar and yet slightly variable in any one species On the other hand the percentage calcification causes the dry density to be lower if the calcification percentage is less at any given

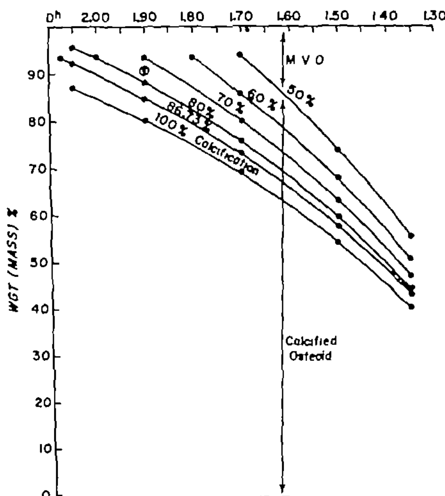


FIG 10-18 Graph showing the points in Table 10-4 The small x circled should be noted at the intersection of the D^h 1.9 line and the 90 per cent mass or weight line on the left of the graph. A specimen of bone lying in this area would have 10 per cent of its weight or mass occupied by the marrow vascular osteocyte space and 90 per cent occupied by the calcified osteoid or bone matrix, and the over all percentage calcification would be about 75 per cent based on our theoretical 100 per cent value. Full calcification would be represented by about 86 73 per cent calcification over-all of this osteoid A.

hydrated sample density All these lines would eventually meet at a point of dry density about 1 4 to 1 45 and a hydrated density of about 1 04 This point represents the situation in which the bone sample would consist entirely of marrow

or the organic matrix can hold 128 per cent of its dry weight as water also it can hold 28 5945/15 7767 = about 181 per cent of its dry volume as water

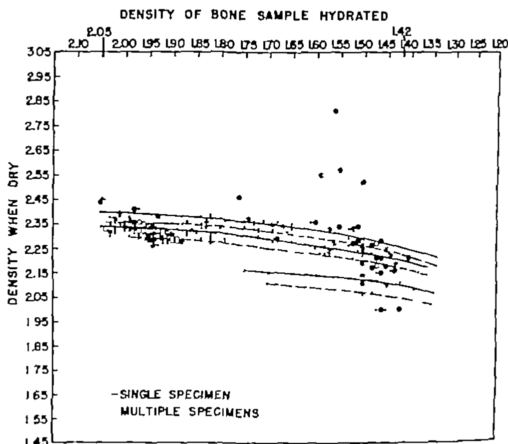


FIG. 10-17 This graph is identical to that seen in Fig. 10-4 except that superimposed on the data points are the curves derived from Figs. 10-20 and 10-23 The solid lines are those obtained from Fig. 10-23 The topmost one represents 100 per cent calcification of the theoretical osteoid B the middle solid line represents 80 per cent calcification of osteoid B and the lowermost solid line represents 50 per cent calcification of osteoid B The topmost dotted line represents 100 per cent, the middle 80 per cent, and the lowest 50 per cent calcification of the theoretical osteoid A.

On the basis of these characteristics for a standard marrow and two similar standard osteoids A and B the following tables were constructed. In Fig 10-17 these two standard patterns are superimposed on density data from 448 bone specimens It is felt that within narrow limits there is probably some difference in osteoids from one dog to another that is the

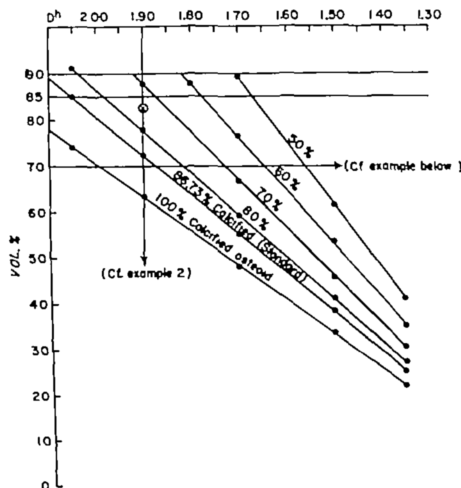


FIG. 10-19 The points on this graph are taken from Table 10-10 and represent volume per cent. There are two horizontal lines across the top of the graph. No lines are extended much above the topmost of these two lines since no bone specimens in the dog apparently have less than about 10 per cent of their volume occupied by marrow-vascular-osteocyte space so that any line projecting above this point is practically theoretical. The 85 per cent line which represents 85 per cent of the volume occupied by calcified bone matrix and 15 per cent occupied by marrow vascular-osteocyte space represents the usual situation found in the specimens of bone from older dogs. Just below these two lines can be seen the *x* which was also observed in Fig. 10-18 at about 75 per cent cal cified matrix in a bone specimen having a hydrated density of 1 9 and a marrow vascular-osteocyte volume of about 18 per cent of the total bone specimen. The two lines (marked with arrows) which cross extending from 1.9 D^h and 70 per cent volume intersect at a point just a little above the standard over-all cal cification percentage of 86 73 In other words a specimen having an osteoid calcified just a little more than average when fully calcified might only have 70 per cent of its total volume occupied by calcified bone matrix, whereas in such a specimen 30 per cent of the volume would be occupied by marrow vascular-osteocyte space A much more realistic situation in view of our findings is the region marked by the circled *x* remarked upon above

Table 10-9

MASS PER CENT MARROW STANDARD OSTEOID A

1.80

D^1	2.05	1 90	1 70	1.50	1.35	Per cent of osteoid which is dry mass
100%						
M V-O	12.8	19 9	31.3	45 0	59.2	100
Osteoid	87.2	80 1	68 7	54 4	40 8	
86.73%						
M V-O	7 6	15 1	27 1	42.4	56.8	90.32
Osteoid	98 4	84 9	72.9	57 6	43.2	
80%						
M V-O	4 4	12.1	24 6	40 4	55.3	94.24
Osteoid	95 6	87 9	75.4	50 6	44 7	
70%						
M V-O		6.8	20.0	37 0	52.6	91.15
Osteoid		93.2	80 0	63.0	47 4	
60%						
M V-O		0.8	14 1	32.1	49 0	86 9
Osteoid			85.9	67 9	51 0	
50%						
M V-O			6.2	26 1	44 4	82.58
Osteoid			93.8	73.0	55 6	

1.80

See Fig. 10-18.

Twelve per cent of M V-O mass is dry mass, having a D^{dry} of 1 45. The D^{dry} osteoid varies because the per cent calcification varies.

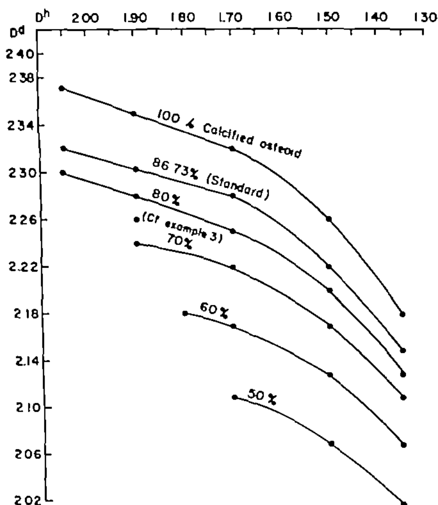


Fig. 10-20 This graph represents the values noted in Table 10-11 A dot is placed between the 70 per cent calcification and 80 per cent calcification lines. In other words a bone specimen having a density when hydrated of 1 9 and a density when dried at 100 C of 2.26 would lie at this point. From such data one would assume that with an osteoid standard A and a standard fat free marrow such as we described, this specimen referring back to Fig 10-19 and Fig 10-18 respectively would have 82 per cent of its volume as calcified osteoid and about 90 per cent of its weight or mass as calcified osteoid and about 18 per cent of its volume occupied by marrow vascular-osteocyte space and about 10 per cent of its mass occupied by marrow vascular-osteocyte space. Subsequent inorganic or organic analyses or both could then in relation to these figures tell us whether the calcified osteoid is calcified normally or subnormally or higher than it should be.

Table 10-10

VOLUME PER CENT MARROW STANDARD OSTEOID A

D^A	2.05	1.90	1.80	1 70	1.60	1.35
100%						
M V-O	25.3	30 4		51.2	65.9	—.0
Osteoid	74 7	03 6		48.8	34 1	23 0
86 73%						
M V-O	15	27 6		44.4	61.2	73 8
Osteoid	85	72 4		55.6	38.8	20 2
80%						
M V-O	8.6	22.2		40.2	58 3	1.9
Osteoid	91 4	77 8		59.8	41 7	28 1
70%						
M V-O		12 4		32 7	53.3	68 3
Osteoid		87 6		67.3	46.7	31 7
60%						
M V-O			11 0	23 1	40.3	63.8
Osteoid			88 1	76.9	53.7	36 2
50%						
M V-O				10.2	37 7	57 .
Osteoid				89 8	62.3	42 3

1.80

See Fig 10-10

The lower the density of the dry osteoid (calcified) or its per cent of calcificatio the smaller the M V-O space at any D^A level

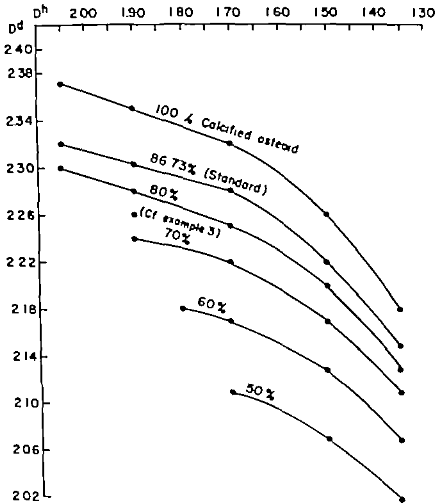


FIG. 10-20 This graph represents the values noted in Table 10-11 A dot is placed between the 70 per cent calcification and 80 per cent calcification lines. In other words a bone specimen having a density when hydrated of 1 9 and a density when dried at 100 C of 2.26 would lie at this point. From such data one would assume that with an osteoid standard A and a standard fat free marrow such as we described, this specimen referring back to Fig. 10-19 and Fig. 10-18 respectively would have 82 per cent of its volume as calcified osteoid and about 90 per cent of its weight or mass as calcified osteoid and about 18 per cent of its volume occupied by marrow vascular-osteocyte space and about 10 per cent of its mass occupied by marrow vascular-osteocyte space Subsequent inorganic or organic analyses or both could then in relation to these figures tell us whether the calcified osteoid is calcified normally or subnormally or higher than it should be

Table 10-11

D^A VS. $D^{d(t)}$ MARROW STANDARD OSTEOID A

D^A	2.05	1.90	1.80	1.70	1.50
100% calcified osteoid	Whole specimen $D^{d(t)}$ 2.37	2.35		2.32	2.26
86.73% (*Standard A*)	2.32	2.3		2.27	2.22
80%	2.3	2.28		2.25	2.2
70%	Not enough space for marrow	2.24		2.22	2.17
60%			2.18	2.17	2.13
50%				2.11	2.07

* See Fig. 10-20

Theoretically all lines should come to the $D^A - D^d$ "cross over" at po
(fat-free) namely D^A – 1 0398 and D^d of 1 4484 or D^A 1 04 and D^d of 1 45.

EXAMPLE If one knows only the D^A or only the volume occupied by t
space in a specimen, one cannot exactly pin point the marrow-vascula
volume or the D^A However if one knows both the D^A and the $D^{d(t)}$ then c
more exact. For instance, the D^A is 1.90 and $D^{d(t)}$ is about 2.26, then one m
the accompanying graph that the per cent calcification of the osteoid is ab
cent. On the mass graph, the weight per cent of the specimen occupied by M V
be about 9.5 per cent while that of osteoid would be 90.5 per cent. On the volu
the volume per cent of the specimen occupied by *M V-O would be about 17.*
while that of osteoid would be about 82.7 per cent.

Table 10-11

D^A vs. $D^{d(b)}$ MARROW STANDARD OSTEOID A

D^A	2.05	1.90	1.80	1.70	1.50	1.35
100% calcified osteoid	Whole specimen $D^{d(b)}$ 2.37	2.35		2.32	2.20	2.18
88.73% (Standard A)	2.32	2.3		2.27	2.22	2.15
80%	2.3	2.28		2.25	2.2	2.13
70%	Not enough	2.24		2.23	2.17	2.11
60%	space for marrow		2.18	2.17	2.13	2.07
50%				2.11	2.07	2.02

* See Fig 10-20

Theoretically all lines should come to the $D^A - D^d$ cross over" at pure marrow (fat-free) namely D^A – 1 0386 and D^d of 1 4484 or D^A 1 04 and D^d of 1 45.

EXAMPLE *If one knows only the* D^A *or only the volume occupied* by the M V-O space in a specimen, one cannot exactly pin point the marrow vascular-osteocyte volume or the D^A However if one knows both the D^A and the $D^{d(b)}$ then one can be more exact. For instance, the D^A is 1.90 and $D^{d(b)}$ is about 2.20, then one may see on the accompanying graph that the per cent calcification of the osteoid is about 75 per cent. On the mass graph, the weight per cent of the specimen occupied by M V-O would be about 9.5 per cent, while that of osteoid would be 90 5 per cent. On the volume graph, the volume per cent of the specimen occupied by M V-O would be about 17.3 per cent while that of osteoid would be about 82.7 per cent.

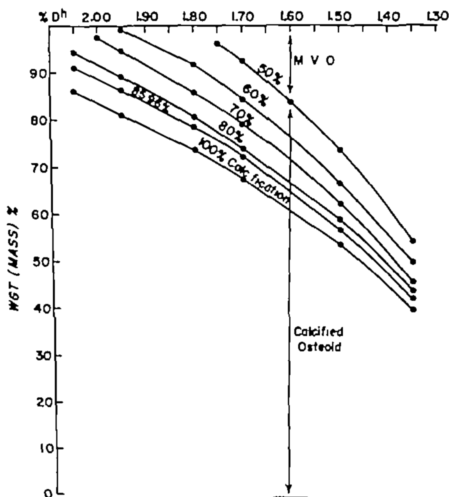


FIG 10-21 This graph correlates the density of the bone when hydrated to it mass per cent division between calcified matrix and marrow vascular-osteocyt space assuming a different percentage calcification of the osteoid B standard

Table 10-11

D^b vs. $D^{s(b)}$ MARROW STANDARD OSTEOID A

D^b	2.05	1.90	1.80	1.70	1.60	1.35
100% calcified osteoid	Whole specimen $D^{s(b)}$ 2.37	2.35		2.32	2.20	2.18
86.73% (Standard A)	2.32	2.3		2.27	2.22	2.15
80%	2.3	2.28		2.25	2.2	2.13
70%	Not enough space for marrow	2.24		2.22	2.17	2.11
60%			2.18	2.17	2.13	2.07
50%				2.11	2.07	2.0[illegible]

See Fig. 10-20

Theoretically all lines should come to the $D^b - D^s$ "cross over" at pure marrow (fat-free) namely D^b – 1.0380 and D^s of 1.4484 or D^b 1.04 and D^s of 1.45

EXAMPLE If one knows only the D^b or only the volume occupied by the M V-O space in a specimen, one cannot exactly pin point the marrow vascular-osteocyte volume or the D^b However if one knows both the D^b and the $D^{s(b)}$ then one can be more exact. For instance the D^b is 1.90 and $D^{s(b)}$ is about 2.26, then one may see on the accompanying graph that the per cent calcification of the osteoid is about 75 per cent. On the mass graph, the weight per cent of the specimen occupied by M V-O would be about 9.5 per cent, while that of osteoid would be 90.5 per cent. On the volume graph, the volume per cent of the specimen occupied by M V-O would be about 17.3 per cent while that of osteoid would be about 82.7 per cent.

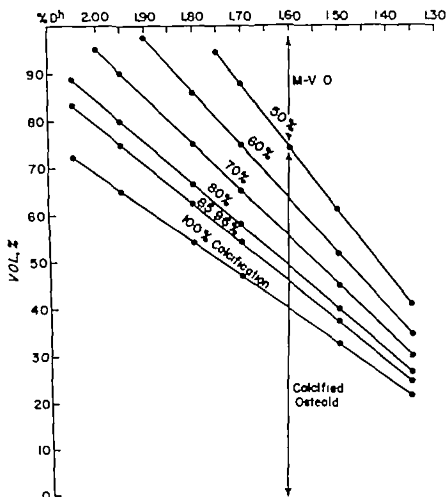


FIG 10-22. The points in this area are taken from Table 10-13 showing the volume per cent division between the standard marrow or standard marrow vascular-osteocyte space and the calcified osteoid standard B assuming different percentage calcifications of the standard osteoid B

Table 10-12

MASS PER CENT   MARROW STANDARD OSTEOID B

D^{s}	2.05	[2.00]	1.95	[1.90]	1.80	[1.75]	1.70	[1.60]	1.50	1.35	*Per cent of osteoid which is dry mass*	*Density of this dry mass*
100%												
M V-O	14		18.5		20.2		32.2		46.4	59.8	100	2.43
Osteoid	86		81.5		73.8		67.8		53.0	40.2		
85.96%												
M V-O	8.5		13.3		21.5		27.8		42.9	57.2	95.99	2.38†
Osteoid	91.5‡		86.7		78.5		72.2		57.1	42.8		
80%												
M V-O	5.6		10.6		19.1		25.6		41.2	55.9	94.08	2.35
Osteoid	94.4		89.4		80.9		74.4		58.8	44.1		
70%												
M V-O		2.5	5.2		14.2		21.1		37.6	53.2	90.50	2.30
Osteoid		97.5	94.8		85.8		78.9		62.4	46.8		
60%												
M V-O				1.2	7.8		15.2		33.0	49.7	86.56	2.24
Osteoid				98.8	92.2		84.8		67.0	50.3		
50%												
M V-O						3.2	7.3	16.4	26.7	45.1	8[illegible].00	[illegible]1
Osteoid						96.8	92.7	83.6	73.3	54.9		

* See Fig. 10-21

Twelve per cent of marrow weight is dry mass. D^{dd} of this dry mass is 1.45.

† Although the $D^{d\,w}$ or density of the dry osteoid not including the water space is 2.38 in this instance, if one includes the water space a lower dry density or D^{d}) is obtained.

‡ For example, hydrated osteoid weight = 91.5. Only 96 per cent of this weight is dry matter, therefore 91.5 × 0.96 = 87.84. Thus 91.5 weight per cent of hydrated osteoid is composed in this instance of 87.84 Gm of dry material having a density of 2.38† and 3.7 Gm of water having a density of 1.0

$$\frac{3.7}{1} = 3.7 \text{ cm}^3 \quad \text{and} \quad \frac{87.84}{2.38} = 36.9 \text{ cm}^3$$

If the sample is dried and correction is not made for the water space, the water space will be filled by air or vacuum which for the purposes of these calculations both have a density of 0.0, then the $D^{d} = \frac{(87.84 - M)}{(40.6 - 1)}$ in other words all the mass is contributed by the dry osteoid but the volume is composed of both the volume of the dry osteoid and the water space. Thus the $D^{d} = 2.16$. *If the D^{d} is determined on bone section* in which canaliculi are present, the D^{d}) will be still lower while the $D^{d\,w}$ of the calcified matrix itself (excluding lacunar, canalicular and osteoid water space) will still be about 2.3

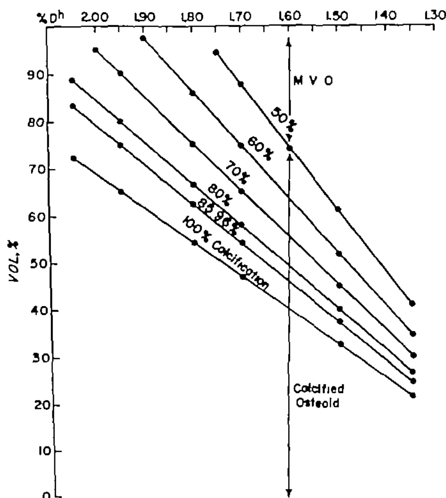


Fig 10-22. The points in this area are taken from Table 10-13 showing the volume per cent division between the standard marrow or standard marrow vascular-osteocyte space and the calcified osteoid standard B assuming different percentage calcifications of the standard osteoid B

Table 10-12

MASS PER CENT MARROW STANDARD OSTEOID B

D^s	2.05	[2.00]	1.95	[1.90]	1.80	[1.75]	1.70	[1.60]	1.50	1.35	Per cent of osteoid which is dry mass	Density of this dry mass
100%												
M V-O	14		18.5		26.2		32.2		46.4	59.8	100	2.43
Osteoid	86		81.5		73.8		67.8		53.6	40.2		
85.96%												
M V-O	8.5		13.3		21.5		27.8		42.0	57.2	95.96	2.38†
Osteoid	91.5‡		86.7		78.5		72.2		57.1	42.8		
80%												
M V-O	5.6		10.6		19.1		25.6		41.2	55.9	91.08	2.35
Osteoid	94.4		89.4		80.9		74.4		58.8	44.1		
70%												
M V-O		2.5	5.2		14.2		21.1		37.6	53.2	90.56	2.30
Osteoid		97.5	94.8		85.8		78.9		62.4	46.8		
60%												
M V-O				1.2	7.8		15.2		33.0	49.7	86.50	2.24
Osteoid				98.8	92.2		84.8		67.0	50.3		
50%												
M V-O						3.2	7.3	16.4	26.7	45.1	82.00	2.17
Osteoid						96.8	92.7	83.6	73.3	54.9		

See Fig. 10-21

Twelve per cent of marrow weight is dry mass. $D^{(s)}$ of this dry mass is 1.45

† Although the $D^{(s)}$ or density of the dry osteoid not including the water space is 2.38 in this instance, if one includes the water space a lower dry density or $D^{(w)}$ is obtained.

‡ For example hydrated osteoid weight = 91.5. Only 96 per cent of this weight is dry matter therefore $91.5 \times 0.96 = 87.84$. Thus 91.5 weight per cent of hydrated osteoid is composed in this instance of 87.84 Gm of dry material having a density of 2.38† and 3.7 Gm of water having a density of 1.0

$$\frac{3.7}{1} = 3.7\ \text{cm}^3 \qquad \text{and} \qquad \frac{87.84}{2.38} = 36.9\ \text{cm}^3$$

If the sample is dried and correction is not made for the water space the water space will be filled by air or vacuum which for the purposes of these calculations both have a density of 0.0 then the $D^{(w)} = \dfrac{(87.84 = M)}{(40.6 = 1)}$. In other words, all the mass is contributed by the dry osteoid but the volume is composed of both the volume of the dry osteoid and the water space. Thus the $D^{(w)} = 2.16$. If the D^{d} is determined on bone sections in which canaliculi are present, the $D^{(w)}$ will be still lower while the $D^{(s)}$ of the calcified matrix itself (excluding lacunar canalicular and osteoid water space) will still be about 2.38.

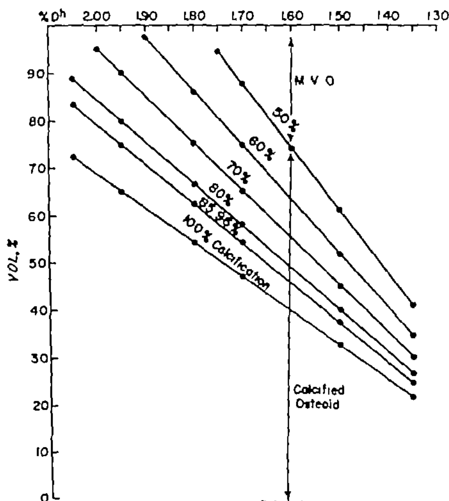


FIG. 10-22. The points in this area are taken from Table 10-13 showing the volume per cent division between the standard marrow or standard marrow vascular-osteocyte space and the calcified osteoid standard B assuming different percentage calcifications of the standard osteoid B

Table 10-13

VOLUME PER CENT MARROW STANDARD OSTEOID B

D^s	2.05	2.00	1.95	1 90	1.80	1 75	1 70	1 60	1.50	1.35
100%										
M V-O	27 0		34 7		45.5		52.6		66.9	77 7
Osteoid	72 4		65.3		54.5		47 4		33 1	22.3
85.90%										
M V-O	16.8		25		37.3		45.6		62 0	74.4
Osteoid	83.2		75		62.7		54 4		38.0	25.6
80%										
M V-O	11 1		19 9		33 1		41.0		59.5	72.6
Osteoid	88.9		80.1		66.9		58.1		40.5	27 4
70%										
M V-O		4 7	9 7		24 6		34.5		54.3	69 1
Osteoid		95.3	90.3		75.4		65.5		45 7	30.9
60%										
M V-O				2.2	13.5		24.9		4 0	64 6
Osteoid				97.8	86.5		75 1		52.4	35 4
50%										
M V-O						5.3	12.0	2.53	38.0	58 0
Osteoid						94 7	88.0	74 7	61 4	41 4

See Fig. 10-22

Table 10-14

D^s vs. $D^{s(b)}$ MARROW STANDARD OSTEOID B

D^s	2 05	2.00	1 95	1 90	1.80	1 75	1 70	1 60	1.50	1.35
100% calcified osteoid	Whole specimen $D^{s(b)}$ 2.40		2.39		2 37		2.35		2.29	2.21
85 96% (Standard B)	2 30		2.35		2.33		2.31		2.25	2.18
80%	2.34		2.33		2.31		2.29		2 23	2.16
70		2.29	2 20		2.27		2.25		2 10	2 11
60%				24	2.22		2.21		2 10	2 10
50%						2 16	2.15	2.14	2.11	2.06

See Fig. 10-23.

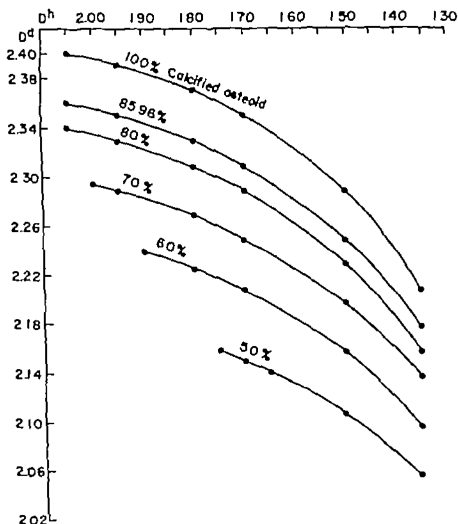


FIG. 10-23 Here the density of the sample when hydrated and the density of the sample when dried are correlated in relation to the standard marrow and admixed with different amounts of standard osteoid B which is variously calcified from 50 per cent through a normal or standard "full" calcification figure of 85.96 per cent to the theoretical 100 per cent calcification level.

Table 10-15

MASS PER CENT WATER DISTRIBUTION STANDARD MARROW OSTEOID A

D^4	2.05	1.90	[1.80] 1.70	1.60	1.35
100%					
M V-O water	11.20	17.51	27.51	40.13	52.10
Osteoid water	0	0	0	0	0
Total water / Dry	11.20 / 88.74	17.51 / 82.49	27.51 / 72.40	40.13 / [illegible]	52.10 / [illegible]
90.72%					
M V-O water	6.69	13.29	23.53	37.31	49.08
Osteoid water	3.40	3.12	2.98	2.12	1.59
Total water / Dry	10.09 / 89.91	16.41 / 83.50	26.53 / 73.47	39.43 / 60.57	51.57 / 48.43
80%					
M V-O water	3.67	10.65	21.65	35.65	48.06
Osteoid water	5.51	5.07	4.35	3.43	2.58
Total water / Dry	9.38 / 90.62	15.72 / 84.28	26.00 / 74.00	38.98 / 61.02	51.24 / 48.76
70%					
M V-O water		5.98	[illegible]	32.50	40.29
Osteoid water		4.25	[illegible]	5.58	4.19
Total water / Dry		10.23 / 89.77	24.68 / 75.32	38.14 / 61.86	50.48 / 49.52
60%					
M V-O water		5.98	12.41	28.25	43.12
Osteoid water		12.14	11.19	8.85	6.64
Total water / Dry		18.12 / 81.88	23.60 / 70.40	37.10 / 62.00	49.76 / 50.24
50%					
M V-O water			5.46	22.97	30.07
Osteoid water			16.34	12.87	9.68
Total water / Dry			21.80 / 78.20	35.84 / 64.16	48.75 / 51.25
Vol*/100 Gm	48.78	52.63	58.82	66.07	74.07

[55.56]

* Volume is found by dividing the component by the figure on the bottom line.

Table 10-10

VOLUME PER CENT WATER DISTRIBUTION STANDARD MARROW OSTEOID A

D^b	2.03		1.90	1.80	1.70		1.50		1.35	
100%										
M V-O water	23.08		33 27		46.82		60 20		70.34	
Osteoid water	0		0		0		0		0	
Total water | Dry	23.08	6 02	33.2	66.73	46 82	53.18	60.20	39.80	70.34	29 66
86.73%										
M V-O water	13.72		25.25		40.54		55.9		67 47	
Osteoid water	0.9		5.93		4.56		3.18		2.15	
Total water | Dry	20.09	79.31	31 16	68.82	45.10	54.01	59 15	40 85	60 62	30.38
80%										
M V-O water	7 93		20.24		36.80		33.325		65.09	
Osteoid water	11.30		9 03		7 40		5 145		3.48	
Total water | Dry	19.23	80 77	29.87	0 13	44.20	55.8	58 47	41.53	69 1	30.83
70%										
M V-O water			11.36		29.92		48.84		62.49	
Osteoid water			8.08		12.04		8.37		5 66	
Total water | Dry			19 44	80.56	41.96	58 04	57.21		68.15	31.85
60%										
M V-O water			10 76		21 10		42 375		58.21	
Osteoid water			21.85		19 02		13.275		8 96	
Total water | Dry			32.61	67.38	40.12	59.88	55 65	44.35	67 17	32.83
50%										
M V-O water					0 28		34 453		52 5	
Osteoid water					27 8		19.205		13.07	
Total water | Dry					37 06	6_.94	53. 0	46.21	65 82	34.18
Wt/100 cm	205.		190	180.	1 0		150.		135.	

Table 10-17

MASS PER CENT WATER DISTRIBUTION STANDARD MARROW OSTEOID B

D^A	2.05	1.95	1.80	1.70	1.50	1.35
	2.00	1.90	1.75	1.60		
100%						
M-V-O water	12.32	16.28	23.00	28.34	40.83	52.02
Osteoid water	0	0	0	0	0	0
Total water/Dry	12.32/87.68	16.28/83.72	23.06/76.94	28.34/71.66	40.83/59.17	52.02/4[illegible]
85.96%						
M-V-O water	7.48	11.71	18.92	24.46	37.[illegible]3	50.34
Osteoid water	3.67	3.48	3.15	2.90	2.29	1.[illegible]2
Total water/Dry	11.15/88.85	15.19/84.81	22.07/77.93	27.30/72.04	40.04/59.96	52.06/47.94
80%						
M-V-O water	4.03	9.33	16.81	22.33	36.26	49.19
Osteoid water	5.59	5.20	4.79	4.40	3.48	2.61
Total water/Dry	10.52/89.48	14.62/85.38	21.60/78.4	26.93/73.07	39.74/60.26	51.80/48.2
70%						
M-V-O water	2.2	4.58	12.5	16.5	33.00	46.85
Osteoid water	9.2	8.95	8.1	45	5.80	4.42
Total water/Dry	11.4/88.6	13.53/86.4	20.6/79.4	26.02/73.98	38.98/61.02	51.27/48.73
60%						
M-V-O water		1.06	6.87	13.38	29.04	43.74
Osteoid water		13.27	12.37	11.39	9.00	6.76
Total water/Dry		14.33/85.67	19.24/80.76	24.77/75.23	38.04/61.96	50.50/49.50
50%						
M-V-O water			2.82	6.42	23.50	39.69
Osteoid water			17.32	16.69	13.19	9.88
Total water/Dry			20.14/79.86	23.11/76.89	36.69/63.31	49.57/50.43
Vol/100 Gm	48.76	51.28	53.56	58.82	60.7	4.07
	50.00	52.63	57.14			

Total water = water lost at 100°C drying to constant weight. M-V-O water = water lost at 50°C drying. Osteoid water = water lost between 50 and 100°C drying.

Table 10-18

VOLUME PER CENT WATER DISTRIBUTION STANDARD MARROW OSTEOID [illegible]

	[illegible]00	1.90	1.75	1.60		
D^h	2.05	1.95	1.80	1.70	1.50	1.35
100%						
M V-O water	25.20	31.7[illegible]	41.51	48.18	61.25	71.01
Osteoid water	0	0	0	0	0	0
Total water|Dry	[illegible]5.20|[illegible]4.74	31.75|68.25	41.51|58.49	48.18|[illegible]1.8[illegible]	61.25|38.75	71.01|[illegible]
85.90%						
M V-O water	15.33	22.84	31.00	41.58	50.03	[illegible].00
Osteoid water	7.32	6.79	5.67	4.93	3.44	2.32
Total water|Dry	[illegible].65|[illegible]14	[illegible]9.63|70.38	3[illegible].73|60.27	40.51|53.40	[illegible]|39.94	70.28|29.7[illegible]
80%						
M V-O water	10.11	18.19	30.20	38.30	54.39	66.41
Osteoid water	11.40	10.32	8.02	7.48	5.22	3.53
Total water|Dry	[illegible]1.57|78.43	28.[illegible]1|71.40	[illegible].88|61.12	45.[illegible]8|54.22	59.61|40.39	[illegible].93|30.0[illegible]
70%						
M V-O water	4.40	8.03	22.50	31.67	49.64	63.25
Osteoid water	18.40	17.4[illegible]	14.58	1[illegible].07	8.84	5.97
Total water|Dry	22.80|77.20	26.38|73.62	37.08|62.02	44.24|55.77	58.48|41.53	69.22|30.78
60%						
M V-O water		2.01	12.3	22.75	43.56	59.05
Osteoid water		25.21	22.27	19.36	13.50	9.13
Total water|Dry		27.22|72.77	34.64|65.37	42.11|57.89	5[illegible].06|42.94	68.18|31.82
50%						
M V-O water			4.91	10.01	35.[illegible]5	53.58
Osteoid water			30.31	28.37	19.79	13.34
Total water|Dry			35.25|64.75	39.28|60.71	55.04|44.96	66.92|33.08
Wt/100 cm³	205.00	193.00	180.00	170.00	150.00	135.00
	200.00	100.00	175.00			

Multiply *water mass per cent* by D^h to obtain *water volume per cent* e.g. 85.96 per cent calcification of matrix and D^h 2.05

M V-O water weight per cent 7.48 (from Table 10-17)
$\times D^h$ 2.05
15.33

Summary and Conclusions

1 It appears that, if one can accurately measure the D^h and the $D^{s(b)}$ of a bone specimen of any one animal species one can estimate its chemical composition as related to the percentage calcification of its matrix and its porosity Estimates of the organic composition may then be checked and

correlated with analyses for nitrogen hydroxyproline hydroxylysine and other components of the organic fraction of a bone specimen Estimates of its inorganic composition may on the other hand be checked by analysis for CO_2, Ca, P Mg and other components of the inorganic fraction of the bone specimen However unless the weights and volumes of the two major divisions of a bone specimen (the marrow vascular-osteocyte space and the osteoid) can somehow be calculated prior to its chemical analysis, no good correlation between the anatomy of a bone specimen and its chemical analysis appears possible For instance, a simple Ca/N ratio tells very little about percentage calcification of the osteoid matrix in a whole bone specimen unless one knows how much of the N in a specimen is associated with osteoid matrix rather than the marrow vascular-osteocyte space

2 The concepts that fat displaces water in the marrow vascular space, that inorganic matter displaces water in the bone matrix or osteoid during calcification, and that bone matrix does not contract or expand during the process of calcification are essential to the method proposed here for correlating chemical analysis and anatomy of bone specimens

3 A corollary to the concept that inorganic matter displaces most of the water in both the cement substance and the collagen fibrils of bone matrix during its calcification is the concept that very little water remains in the matrix, after full calcification has been achieved for the migration of ions (such as calcium and strontium ions) that move through bone matrix in a hydrated state. Thus the rate of movement of such ions would seem to be sharply diminished after the initial phase of calcification

4 Electron micrographs of bone and other normally calcifying tissue matrices contribute certain concepts which are of use in the organization of the chemical analysis data of bone The first of these is that the collagenous organic matrix of bone is present prior to calcification and does not disappear during calcification Secondly the inorganic matter forms not only on the fibrils and between them but in them as well Thirdly calcification of the matrix occurs in conjunction with the collagen in fibril form. Fourth, calcification occurs rather abruptly in bone matrix in the normal animal

5 Bone porosity per se is no indication of incomplete calcification of the bone matrix actually present in a specimen of porous bone

Acknowledgments

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11

Ultrastructure of Bone Mineral

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Despite the seemingly simple chemical composition of the inorganic fraction of bones and teeth and despite the fact that since De Jong s early x ray crystallographic studies in 1926 considerable efforts have been made to clarify the molecular structure of the bone salt, we are today unclear on several points It has been not only the bone salt itself which has evoked such controversial opinions, but also a group of calcium phosphate minerals such as dahllite francolite and staffelite

It was early recognized that the bone mineral was an apatite with a crystallographic structure similar to that of the pure mineral fluorapatite The crystallographic structure of this compound was worked out by Náray Szabó[1] and Mehmel[2] independently in 1930 their proposed structure is in the main correct, although slight adjustments of the positions of the individual atoms have subsequently been made In contradiction to the bone apatites and the so-called carbonate bearing apatite minerals fluorapatite is well crystallized and suitable for precise x-ray crystallographic studies The cause of disagreements regarding the structure and composition of the bone and dental apatites as well as of the carbonate bearing apatites is to be sought in the minute crystal size in the compounds Various types of net chemical formulas have been proposed as well as various types of defects, but it may be relatively safe to state from the beginning that the major inorganic component in bone and the minerals of the staffelite type is essentially pure hydroxyapatite

Figure 11 1 shows the basal projection of fluorapatite and the fluorine atoms are located in the corners.[1, 2] The hexagonal unit cell has the dimensions $a = 9\ 370$ A and $c = 6\ 884$ The space group is C_{6h} ($C6_3/m$) There are two types of calcium ions involved Ca_I which centers around the F or OH ions in the corners of the unit cell and the columnar calciums

Ca_{II} Certain investigations tend to show that the reactivity of these two types of calcium ions is slightly different The axial dimensions of pure hydroxyapatite are $a = 9.421$ and $c = 6.882$ and the hydroxyl ions are substituting the fluorine ions

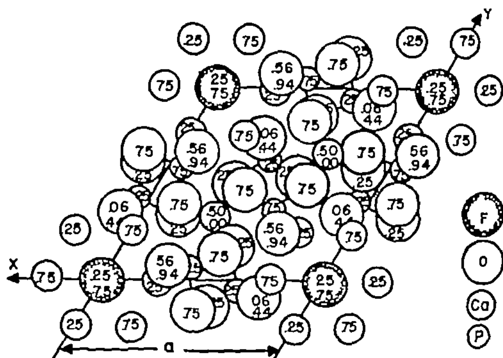


Fig 11 1 The lattice of fluorapatite projected on the basal plane (*Carlström D and Engström A In Bourne G H ed., "The Biochemistry and Physiology of Bone" Academic Press Inc New York* 1956.)

The general confusion brought into the field of apatites by *McConnell*[1,2] with his "carbonate apatites," and by Dallemagne with his α-tricalcium phosphate and also the proposal of Dallemagne that the bone apatite has large defects all seem to have been resolved and can be explained on the basis of the minute crystallite size which is in the order of 10^{-3} to 10^{-7} cm It is, however not definitely proved or disproved that there are minor defects or strain in the hydroxyapatite crystallites, a circumstance which is being carefully investigated by Dr D Carlström at the Department of Medical Physics, Karolinska Institutet Stockholm[3]

The small crystallite size of bone and dental apatite is clearly demonstrated by the broadening of the x ray diffraction reflections at wide angles (Fig 11 2 17) The same finding although to a smaller extent holds for the "carbonate-bearing apatite minerals," and although these can be obtained in large transparent pieces resembling fluorapatite, it has not been possible to record a single crystal x ray diffraction pattern If a thin piece of francolite for example is examined with the flat film technique (Fig 11 3)

there is always an arching of the reflections indicating a relatively small crystallite size but a good preferential orientation of these crystallites

Because of the small size of the crystallites of the bone apatite which results in broadened reflections the lattice parameters cannot be measured with high accuracy the dimensions are $a = 9\ 42 \pm 0\ 03$ and $c = 6\ 88 \pm$

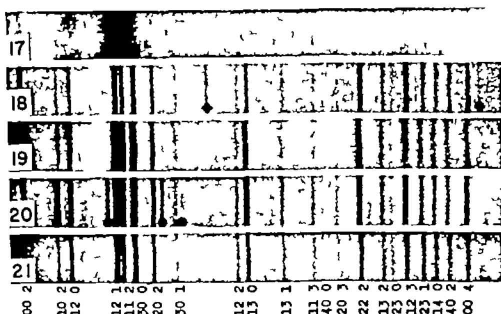


FIG. 11 2. Wide-angle diffraction patterns of various apatites. (17) Untreated bone. (18) Human bone heated to 900 C. Squares indicate CaO-reflections (19) Untreated human enamel Note the sharpness of pattern. (20) Human enamel heated to 900 C Circles indicate 3-tricalcium phosphate (21) Synthetic hydroxyapatite (*Carlström D and Engström A., In Bourne G H ed The Biochemistry and Physiology of Bone" Academic Press Inc New York* 1956)

0 01 On heating, the diffraction lines do become sharp but there is the possibility of transformation of the lattice depending upon the nature of ions adsorbed to the apatite matrix. Thus if there is a surplus of phosphate ions, a β-tricalcium phosphate pattern is obtained after heating which appears superimposed on the apatite pattern (Fig 11 2 20) If there is a surplus of basic groups however CaO lines appear in the diffraction pattern (Fig 11 2.18) A peculiarity is that the a axis of pure hydroxy apatite contracts after heating and also the a axis of enamel becomes 0 01 A after heating to 900 C The c axis does not seem to change its dimensions as far as can be measured.

Before further discussing the properties of the bone apatite it may be of interest to investigate what configuration other types of calcified structures possess Most pathologic calcifications in the human body such as ectopic bone calcified tuberculous loci phleboliths, arteriosclerotic calcifications capillary calcifications etc all give an x ray diffraction pattern similar to that of bone indicating their apatite nature and the minute size

of crystallites Urinary concretions vary greatly in their chemical nature, but those containing hydroxyapatite give diffraction patterns indistinguish able from bone A remarkable finding is that β-tricalcium phosphate is sometimes found in dental and salivary gland calculi. A peculiar circum stance is that the otoconia or statoliths consist of relatively well-crystallized pure calcium carbonate in man and warm-blooded animals in the

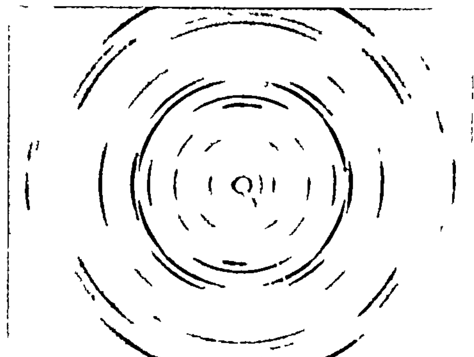


FIG. 11 3 Flat film x-ray diffraction pattern of a thin piece of francolite.

crystallographic form of calcite. In cold-blooded animals the $CaCO_3$ exists in the form of aragonite There are however certain exceptions to this rule the shark, for example has calcite in its otoconia. Some primitive fishes have hydroxyapatite in an almost completely amorphous form in their otoconia.

The exoskeleton of crustaceans consists of calcium carbonate, in lobster for example in the form of calcite The degree of crystallinity varies from one point to another in the lobster shell. The organic framework in this case is a chitin, but there is no information yet available as to whether there is any structural relationship between the organic and inorganic fraction As the crystal structure of chitin is known in detail such studies would seem rewarding when it comes to explaining the relationship or interaction be tween the two components involved.

Returning to the structure of bone apatites, one must bear in mind that samples taken e g from human bone for x ray diffraction studies are inhomogeneous from a micromorphologic point of view as illustrated in

Fig 11-4 When a piece of bone is taken for investigation with the x ray powder technique or a slab is cut out for examination with the fiber photograph procedure, usually Haversian systems of varying degree of mineralization, cementing lines, cortical bone etc. are included As a result the x-ray investigation can be expected to represent only the mean structure of several inhomogeneous structures which represent different stages of growth and development



FIG. 11-4 Microradiogram of a cross section of human femur indicating the varying degree of mineralization.

Therefore a priori, it looks as if the x ray examination of the thin long bones from fish, the phalanx from bat etc would offer special opportunities as they appear rather homogeneously mineralized The marked arching of the reflexes indicates a high degree of orientation, in fact better than in any other type of bone (Fig 11 5 7) Carlström has succeeded in orienting such specimens in the x ray diffractometer in such a way that the diffraction line profiles of the 00'2 and 00'4 reflections can be accurately measured. In other words, it is possible to avoid disturbances from neighboring lines by making up a composite oriented specimen of fish bones.

The line profiles (Fig 11-6) of the bone salt have hitherto been measured only for the 00'2 reflection which gives the length along the *c* axis After Fourier analysis of the 00'2-line profile fresh bone gives a mean value of about 220 ± 30 A If this value is correct, it should naturally

mean that a certain fraction of the specimen consists of particles of this length, but it does not exclude fractions of shorter or longer particles

It was not until recently that Carlström[3] succeeded in measuring the line profile from a *hko* reflex in an oriented fish-bone specimen, which gives a measure of about 40 A for the extension along the *a* axis Naturally as deduced, a fairly homogeneous cross section does not mean a rod with a

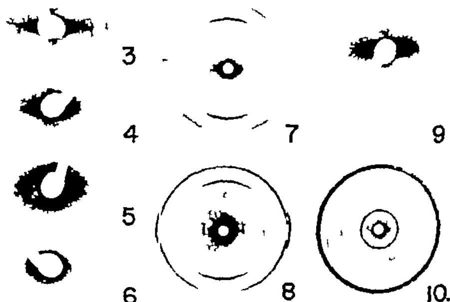


FIG. 11 5 X-ray diffraction of fish bone. (3) Low angle diagram of untreated fish bone. Fiber axis vertical (4 5 and 6) Fish bone heated to 200 300 and 400 C for 1 hour respectively (7) Wide-angle diagram of untreated fish bone. Fiber axis vertical. (8) Wide angle diagram of fish bone heated to 700 C for 1 hour (9) Low-angle diagram of fish bone after removal of the collagen (10) Wide angle diagram of heated fish bone. Direction of x-ray beam parallel with fiber axis. (*Reproduced by permission of Biochim et Biophys Acta* 13 186, 1954)

circular square or hexagonal section one dimension in the cross-sectional area could well be about two times the other although this latter possibility seems less likely

Regarding the length it is difficult to state a precise value and the fact that the extension along the *c* axis as measured from the 00′4 line broadening gives a smaller value from that of the 00 2 line has led Carlström and Glas[4] to propose relatively long particles (about 600 A) having a kinked structure For details, see the recent communication by Carlström and Glas [4]

Still more complicated is the interpretation of the low-angle scatter from bone which is of the gas-solid diffuse type[8, 9] The origin of this type of scatter is due to the difference of electronic density between the particles

and the matrix in which they are embedded It is safe to assume that the scatter is a measure of the particle size in dilute solutions but as the system becomes more and more closely packed, interfering phenomena appear In a very closely packed system the low angle scatter is a measure of the holes rather than the particle size of solid phase Now an oriented bone specimen gives a wing shaped scatter, with the wing perpendicular to the long dimension of the bone (Fig 11 5 3) This means that the units responsible for the scatter are elongated with the long axis parallel to the

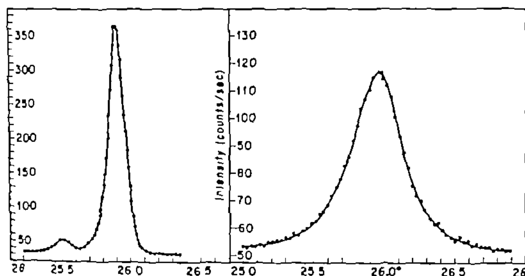


FIG. 11-6 Diffraction profiles for the 002 reflection of fluorapatite and bone.

long axis of the bone. If a slope analysis (Fig 11 7) is made according to the method given by Guinier a fairly straight line is obtained (that is, log scattered intensity versus the square of the scattering angle) and it gives a short dimension of the scattering unit of 50 to 70 A. This value is obviously somewhat too high, as it is impossible to get a perfectly oriented specimen of bone Therefore this value contains some contribution from the long axis and a correction for the orientation would give the value 40 to 50 A for the corrected width. In the same way the long dimension can be estimated to more than 200 A but this value contains a large error

The question is do these dimensions derived from the low-angle scatter represent the apatite particles or the space between them? The latter interpretation has been put forward by Caglioti et al.[7] based on the argument that collagen gives such a wing shaped diffuse scatter at low angles This collagen diffuse scatter however is considerably less intense and the fact that these authors base their interpretation on electron micrographs of exceedingly poor quality makes their interpretation less valid. Furthermore bone is not such a closely packed system as would give a measure of the holes rather than of the particles an opinion supported by studies of hydroxyapatite precipitates

Although it cannot be absolutely stated that the low-angle scatter arises from the particles a number of observations speak in this direction Pure synthetic apatite with a wide-angle diffraction pattern similar to that of bone gives identical scatter for a wide range of dilutions where obviously the interparticle distance varies. When bone is heated (Fig 11 5 4-6) the low angle particle scatter disappears, which means larger particles as observed also by the change of the wide-angle x ray diffraction pattern (Fig

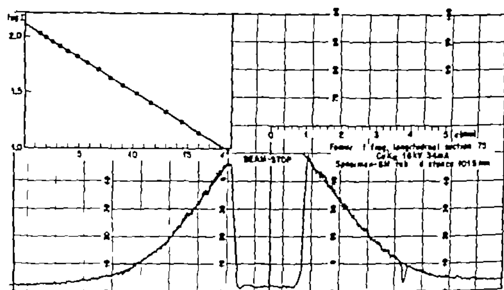


FIG. 11 7 Intensity distribution in the low angle scatter corresponding to the short dimension of the crystallites. (*Upper left*) Log scattered intensity versus the square of the scattering angle.

11 5 8 10) It is certainly true that the low-angle scatter becomes somewhat more intense when the organic phase is extracted (Fig 11 5 9) but this is to be expected as the difference in electronic density between the two phases becomes larger

Thus the results of the low-angle scatter is in a general way in agreement with the results of line profile measurements in the wide-angle diagram and one is tempted to ascribe the scattering effect to the particles, especially as bone is not such a closely packed system with regard to the inorganic phase as would be required to give the size of "holes" With refined x-ray crystallographic techniques, we are reexamining these phenomena also from the point of view that it is obvious from other measurements of peak and diffuse scatter that the original intensity distribution as predicted by the Guinier theory is not quite valid.

The recent results obtained by the electron microscopy of bone tissue seem to agree in general with the deductions presented above. Thus the apatite particles appear elongated with a width of about 20 to 50 A.[a, b]

The length is somewhat variable, but it would appear safe to state that the apatite particles have a considerable extension

Both the x-ray crystallographic and the electron microscope investigations indicate a close relationship between the organic matrix collagen and the mineral phase This was observed as early as 1923 when W J Schmidt examined bone in the polarizing microscope He expressed the

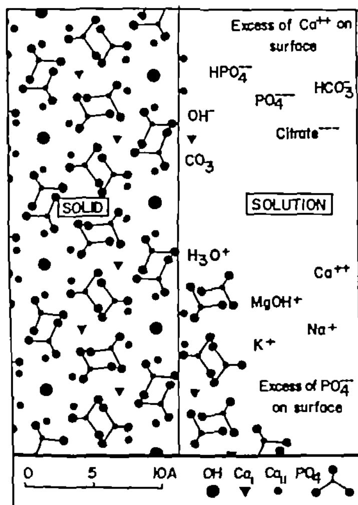


FIG. 11-8 The boundary of an apatite crystallite (*Courtesy of D Carlström Acta radiol Suppl.* 121 1–59 1955)

view that the collagen fibers had the ability to adsorb the apatite particles in an oriented way It is not possible at the moment to decide whether there is a linkage between the organic matrix and the mineral phase or if the orientation of the collagen fiber network physically forces the apatite particles to align themselves along the fibers More information about the precise structure of the bone collagen is needed, but unfortunately it is necessary to treat bone in a relatively drastic way to study the bone collagen itself In certain tendons of birds calcium salts are deposited in an oriented

way that is the apatite particles are aligned between the collagen fibers. The calcification front is relatively sharp and later on resorption and rebuilding take place in other words, the calcified structures are being transformed into bone During this procedure the parallel structures are somewhat distorted. In the case of the calcified tendon, studies in polarized light, micro x-ray diffraction and microradiography tend to indicate that the collagen fibers in some way "adsorb" and align the apatite particles. In the ossified part of the tendon, the conditions are more complicated and the problem has to be studied further

The small size of the apatite particles means that they have a large surface area which can adsorb a variety of ions (Fig. 11-8) An interesting question is, what limits the growth of the particles? At first sight one would perhaps tend to believe that the organic matrix has some limiting function. On the other hand, do all pathologic calcifications, which occur without organic matrix, have about the same crystallite size as that in bone and dentin? One is tempted to speculate that the carbonate which is surface *bound and probably incorporated by epitaxy can be a stopping factor* If this is the case the crystallites should stop growing when a certain number of such defect areas are present and if this is the case we have to consider the bone apatite as some sort of a defect structure The fact, however that enamel has much larger crystallites under approximately the same ionic conditions complicates the picture It is not possible at the moment to give an unequivocal answer to these questions.

When studying the substituted apatites, it appears that it is difficult, under the circumstances which are present in the organism, to introduce other positive ions in the lattice For example rats were continuously fed with strontium instead of calcium and, when sacrificed, the bones contained about 10 per cent strontium as determined by x ray analysis. The length of the crystallographic axes was exactly that of pure hydroxyapatite If strontium had entered the lattice, there would have been a shift in the axial dimension, as strontium hydroxyapatite has $a = 9\ 761$ A and $c = 7\ 277$ A as compared with $a = 9\ 423$ A and $c = 6\ 884$ A for hydroxyapatite The conclusion is therefore that strontium is mainly bound by surface adsorption and eventually incorporated by epitaxy In fact, experiments performed in order to try to precipitate mixed calcium–strontium apatites showed that it was very difficult to introduce larger amounts of strontium in the hydroxyapatite lattice in the wet way Only the end members of the series $Ca_{10}(PO_4)_6(OH)_2 - Sr_{10}(PO_4)_6(OH)_2$ were able to incorporate both positive ions.[10] When bone is heated in the presence of Mg, there is a contraction of crystallographic axes This observation of course has some pertinence on the axial dimensions of the mineral apatites. Carlstrom found that there was no correlation between axial dimensions and carbonate content, but rather indicated a correlation with the content of fluorine and magnesium Thus it appears that, also in these carbonate-

bearing apatite minerals, the carbonate is surface-bound to the crystallites or occurs as a separate amorphous phase

Although many questions still have to be answered regarding the structure of the bone mineral certain main features are well established Bone apatite is hydroxyapatite the sizes of the crystallites are small they are elongated and about 30 to 50 A in width and several hundred (~600) angstroms long It should be mentioned however that because of the inherent characteristics of our methods of investigation the values of crystallite size is a mean value or a value selected from a population depending on the inherent nature of the method used for the measurements What one really would like to give is the *range* that is the distribution of crystallite sizes in various microscopically homogeneous bone structures This is not possible with the present methods It is also safe to state that there is close coordination between the extension of the collagen chains and the direction of the apatite crystallites although the precise nature of the mechanism responsible for this arrangement is not known at present.

Acknowledgments

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FIG. 12 1 Autoradiograph of a transverse ground section of the radius from an adult dog which had been injected with Ca^{45} 7 days before sacrifice

A reliable analysis of such an autoradiograph requires reference to the micro-radiograph and to the histology of the section (Fig 12 2) It is then possible to account for the variety of imprints in terms of subjacent calcification processes. (×16.5)

observations which give us a deeper insight into the life of bone, that is, into the phenomena which are subjacent to the autoradiographic picture

Figure 12 2A shows the very section which had given the autoradiograph as it appears when treated without decalcification by the periodic acid–Schiff (PAS) method It demonstrates the successive stages of the formation of an osteon Of course the region had to be selected in order to present all the interesting features in the same field

In *a* there is a resorption cavity that is the section with an irregular and festooned outline of a tunnel which is bored longitudinally in the diaphysis

In *b* we observe the filling of the cavity operated by the concentric laying down of successive layers of bone The deposition occurs in two steps formation of a PAS-positive layer (arrow) and its subsequent transformation into a PAS-negative layer The innermost layers just elaborated are separated by a dark line from the surrounding layers, laid down previously The PAS method as applied here to undecalcified ground sections, is an easy one to demonstrate the succession of two phases which

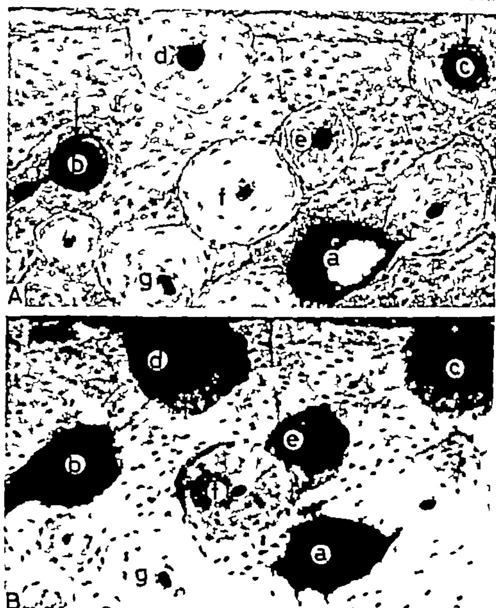


FIG 12 2. *A* Transverse ground section of the radius of an adult dog (the very section which had produced the autoradiograph of Fig 12 1) Treated by PAS method without previous decalcification. (×165) *B* Microradiograph of the same section or map of its calcium content. The information brought by the x rays may be transferred to the histologic picture with the aid of the letters. (×165) (*Reproduced by permission of R Ponlot Arch biol., Par.*, 69 441–454)

had escaped attention until recently Except for the delicate cementing lines, delineating the osteons from the interstitial lamellae, like the cement of a mosaic, the inner ring we speak of is the only structure to contrast in pink against a pale background

In *c* more lamellae have been deposited and the inner ring (arrow) is

somewhat thinner In *d* and in *e* the deposition is nearly complete there is still, however a very thin inner ring hardly discernible at this magnification In *f* and in *g* two fully deposited osteons with narrow Haversian canals seem identical although they are not as will be proved very soon

The section just examined under the microscope had been microradiographed with soft x rays on a fine-grain emulsion Figure 12 2B (with its letters added as in Fig 12 2A) represents the now familiar picture Nearly every cell of Fig 12 2A finds its counterpart in a black dot of Fig 12 2B and correlation is therefore justified at least down to the level of the size of a cell.

With the kind of x rays that we have used the microradiograph is a map of the calcium content of the tissue [1] A section whose organic matter was removed would give the same picture and a decalcified section would produce a uniform faint shadow The whiter a structure appears the more calcium it contains

By transferring the information of the microradiograph to the histologic picture some important remarks emerge at once

The innermost layers of the depositing osteons (arrows of Fig 12 2A) are not seen on the microradiograph Their calcium content is too low to absorb the x-rays used in this case They would show up however with softer x-rays All this means that bone tissue appears first as a preosseous layer" Intense calcification keeps pace but follows in the wake of osteogenesis after a noticeable delay

By photometric measurements [2] it can be estimated that, when it begins to calcify heavily (osteons *b* and *c* in Fig 12 2A and 12 2B) the bone is loaded all of a sudden to at least three-quarters of its full capacity Afterward, calcification proceeds much more slowly Deposition of lamellae is about to end in osteons *d* and *e* whose calcium content is hardly superior to that of osteons *b* and *c* By the time the deposition of lamellae has ceased, calcification has still to be completed Osteogenesis has come to an end in osteon *f* which must still further calcify before its calcium content reaches that of osteon *g*

In reference to our main interest, the metabolism of calcium at the histologic level the transformation of the preosseous layer into real calcified bone (even if this bone is not yet fully calcified) is indeed critical and deserves supplementary comments

The preosseous layer incorporates radiosulfur [3-7] With toluidin blue the preosseous layer and the outer layers of the same depositing osteon react differently [4, 5, 7, 8] in our slides the former is orthochromatic whereas the latter is metachromatic. Methylene blue extinction occurs at pH 4 in the preosseous layer and at pH 5 6 in the outer layer [7] The Gomori reaction for alkaline phosphatase stains only the preosseous layer and not the surrounding layers at all except for the cells

It should also be added here incidentally that decalcified sections re

calcify in vitro under certain conditions [10] Recalcification occurs only in the osteons with a low calcium content, that is in the osteons which were calcifying in vivo

Recently Irving[11] has noticed that Sudan black B stains the areas where calcification was being initiated, if the section had been previously extracted with pyridine Applied to compact bone,[12] which had not been tested yet, the method gives results which are worth being put on record. Figure 12 3A represents a depositing osteon (identical with osteons *b* and *c* in



Fig. 12 3 *A* Aspect of a depositing osteon in a section extracted with pyridine and treated with Sudan black B (×290) *B* Microradiograph corresponding to *A* Arrows pointing to the same cells have been added to *A* and *B* to convince the reader that each illustration may be safely superimposed on the other (×290) (*Reproduced by permission of R Ponlot Bull micr appl* 8 125–126, 1958)

Figs 12 2A and 12 2B) treated by Sudan black B Figure 12 3B is the corresponding microradiograph. Arrows pointing to cells are there to convince the reader that both illustrations may be exactly superimposed on each other Sudan black B stains specifically a layer which is certainly about to calcify heavily It would even seem in agreement with Irving's observations on other materials that the method detects the beginning of calcification which does not appear in our x ray picture What matters here is the demonstration that the fixation of calcium to the amount of about three-quarters of the capacity is a very fast process indeed.

All these data obtained without the use of Ca^{45} would be better linked with each other if we could evaluate exactly the rate of the Haversian remodeling. The subject has been thoroughly studied by Vincent.[13] By following the fate of S^{35} from week to week at the histologic level he has

established that it takes 6 weeks for an osteon of average size to be deposited and that 12 additional weeks are not always enough for this osteon to complete its calcification

More recently a significant detail has been added Lead is a useful tool in this type of research because it follows the metabolism of calcium and it can be detected histochemically [14] A dog is given lead acetate with the food then returned to normal diet. Twelve days after the beginning of the experiment, it is again given lead and sacrificed a few hours later Figure 12-4 is an example of the result It is partly schematic On the real



FIG 12-4 Lead follows the metabolism of calcium and can be detected histochemically It is therefore a useful tool to evaluate the rate of the Haversian remodeling. (×160)

photograph the preosseous layer has been painted in white Thick concentric lines delimiting a shaded area represent the beginning and the end of the experiment. The cementing lines have been accentuated Looking at osteons *a* *b* and *c* we observe that, once started, the deposition of lamellae is a process which slows down progressively Much more ought to be said about Fig. 12-4 Compared with others osteon *z* indicates that the progressive slowing down seems related to the size of the canal to be narrowed For us the bare fact is important because obviously the amount of calcium retained by a depositing osteon during a given period of time depends on the number of lamellae deposited during this time

We are now in a better position to analyze the Ca^{45} autoradiographs. First of all we are well aware of the subjacent calcification processes From

the technical point of view we realize that the study requires the comparison of every spot of the autoradiograph with both the corresponding microradiograph and the section itself Practically the ground section which has produced the autoradiograph is *microradiographed and then treated,* without decalcification, by the PAS method. The three pictures are examined together with the aid of three identical microscopes projecting their images close to each other on the same screen

In animals sacrificed some days after injection, the densest imprints are those of the osteons which are beginning their deposition From them, down to the weakest, the imprints are seen to correspond to osteons about to end their deposition or to osteons which are completely deposited but not yet fully calcified.[13] On the whole the localized imprints are like a snapshot of the subjacent calcification processes.

In animals sacrificed some weeks after injection the initial picture is still present. But a few weak ring-shaped imprints have been added they belong to osteons which have initiated their deposition after injection, when the specific activity of the blood had fallen.[14-17]

Let it be recalled here that the specific activities of the densest imprints have been recently[18] estimated by densitometry to range up to 35 times that of the diffuse reaction.

Cancellous Bone. Space does not permit an adequate treatment of what we know on cancellous bone[8, 13, 19-23] Generally speaking, *histology* and microradiography indicate that cancellous bone is fundamentally similar in many respects to compact bone The specific activity of the extremities of an adult long bone is two or three times higher than that of the diaphysis, and the ratio does not change much in a period of about 200 days. Furthermore, as revealed by autoradiography the radioactivity of an extremity is the sum of two activities that of the epiphysis, which is low and that of the *metaphysis which is very high. In other words* the adult long bone keeps in its extremities something of the differences between epiphysis and metaphysis which prevail during growth. The reasons for this distribution are still obscure at least in my mind

The (Bone

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to the regions of bone growth. (The section in this particular case has missed the small lower epiphyseal center.)

A detail needs to be investigated at once. What is the exact counterpart of the very dense lines indicated by two arrows? Do they belong as we may expect from silver methods[23, 24] and from electron microscope findings[25, 26] to the hypertrophied cells of the growth cartilage which are

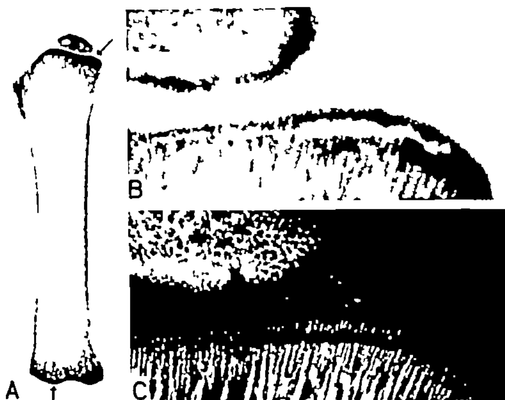


FIG. 12.5. *A*. Autoradiograph 5 hours after Ca^{45} injection of a longitudinal section of the tibia in a 3 week-old dog. The radioactivity belongs to the regions of bone growth. Arrows point to dense lines which are identified by *B* and *C* (×2). *B*. Enlargement of a similar autoradiograph which happens to present a crack—serving as a fiducial mark—in the upper dense line of *A* (×27). *C*. Microradiograph corresponding to *B* and showing that the dense line of the autoradiograph is produced by the calcified layer of the growth cartilage (×27).

calcifying? The autoradiograph of another similar animal happened to present in its upper extremity an opportune crack (Fig. 12.5B) which may serve as a fiducial mark when compared to the corresponding microradiograph (Fig. 12.5C). The latter, confirmed by microscopic examination of the specimen itself, proves that the dense lines which are so obvious in Fig. 12.5A are produced by the hypertrophied cells of the growth cartilage. The other details of the extremities in Fig. 12.5A will become self-explicit when followed in later stages.

Puppies of the same litter were injected at the age of 3 weeks. They

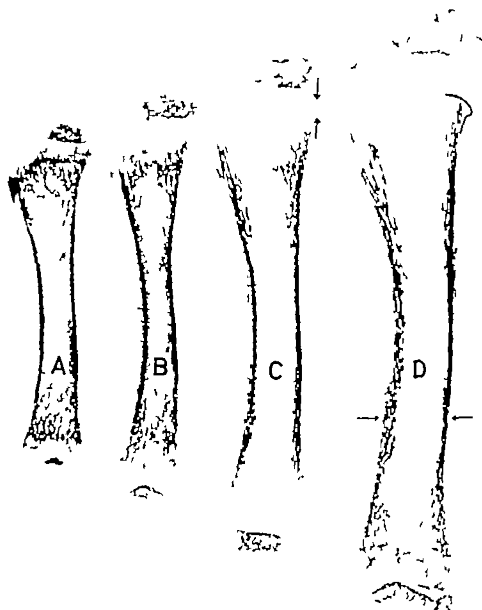


FIG. 12-6 *A* Autoradiograph of the tibia recorded 1 week after Ca^{45} injection in a 3 week-old dog. Growth of the extremities is apparent. (×2.3) *B* Same as *A* but recorded 2 weeks after injection. The mode of growth of the extremities is now fully detailed. (×2.3) *C* Same as *A* but recorded 3 weeks after injection. The distribution of transverse growth in the diaphysis is unequal (×2.3) *D* Same as *A* but recorded 4 weeks after injection. At the level of the arrows, the posterior wall of the bone has been left undisturbed by the remodeling. On this illustration part of the contour of *C* has been added *to show that the metaphysis projecting between arrows in C has been resorbed* in 1 week. The radioactivity of the bone which has been destroyed is redistributed in the newly formed bone. (×2.3)

were then sacrificed at weekly intervals Four autoradiographs are presented They are marked from Fig 12-6A for the 1 week stage to Fig 12-6D for the 4-week stage

When considering the extremities, a number of statements do not need much comment. Pushed by the functioning of the growth cartilages the epiphyses migrate from the strongly labeled tissue Growth in length of the tibia in these animals is equal at both extremities As shown by the upper extremities in A and B the epiphyses develop along the radii of a sort of hemisphere whose flat surface is the growth cartilage The tibial tuberosity migrates apace with the upper epiphysis an apparent migration of course which is the result of an addition of new bone at its upper aspect, where the patellar tendon inserts and of a destruction at its lower aspect On D part of the contour of C has been added It helps to prove that the bone between the two vertical arrows in C has been resorbed in 1 week the histology of this process is illustrated elsewhere [27] All this had been established without the isotopes,[28] but now instead of elaborate demonstrations or indirect evidence a few autoradiographs are all we need to be convinced

One aspect, however of these phenomena cannot be observed with classical methods [29] The new endochondral trabeculae are faintly labeled the less so the more recent they are Most of their Ca^{45} comes from the bone labeled at the time of injection and which is being destroyed Confirming what Hevesy[30] had recorded by specific activities measurements our autoradiographs show that a large part of the calcium of the old bone is being reused by systemic redistribution in the new bone As time goes on less and less initially labeled bone is destroyed, and more and more new trabeculae are produced This is why there is a gradient of diminishing radioactivity from the older to the newer postinjection bone Carried by the blood stream from the destruction sites to the building sites some Ca^{45} is, in passing, excreted by way of the kidney or bowel Because it forms more bone than the adult the growing animal retains initially more Ca^{45} per unit weight of its skeleton But the latter has more opportunities to dispose of its Ca^{45} than the adult. What the young animal retains is redistributed throughout the growing skeleton, always more "diluted" as time goes on

The Diaphysis. Figure 12-6 is of value also to visualize the evolution of the diaphysis Comparing A with D we see that, in 4 weeks the anterior wall has been almost completely destroyed and replaced by a new one farther forward. The posterior wall has been much less remodeled. The contrast between the two walls is evident in D at the level of the arrows where the posterior wall still contains some elements which, 4 weeks before were the highly radioactive structures located in the growth cartilage and in the metaphysis Each individual bone has its own portion comparable in stability for example in the humerus it is a limited area of its medial wall

At first sight, the detail seems trivial. In fact, if we think that Sr^{90} behaves like Ca^{45}, it appears that these small parts which are left undisturbed by the remodeling, and which remain relatively stable are subjected to a much more intense and prolonged irradiation than the rest of the ever changing structure. The progressive "dilution" of the radioactivity in the growing skeleton of which I have just spoken, is not generalized, and the exceptions to the rule are sufficient to warn against undue optimism.

The stability of some parts of a growing long bone is a notion which would

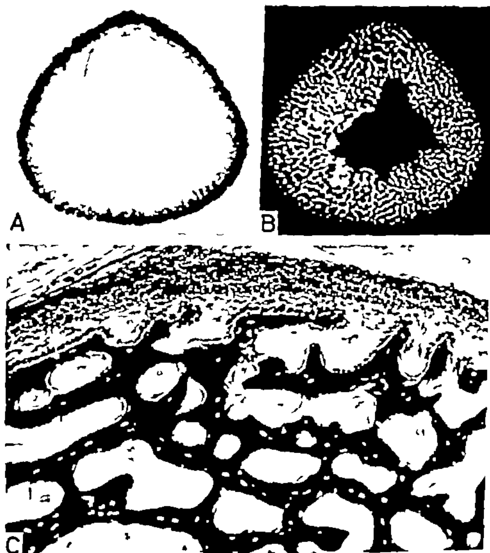


FIG. 12.7. *A.* Autoradiograph recorded from a transverse section of the tibial shaft of a newborn puppy which had received Ca^{45} 24 hours before sacrifice. (×17) *B.* Microradiograph of the section from which the preceding illustration was obtained. Radioactive bone is found only at the periphery of the shaft. (×17) *C.* Histology of a corresponding area making the combination of the three illustrations self-explanatory. Osteogenesis occurs only under the periosteum and there is no sign of Haversian remodeling deeper in the shaft. (×183)

gain if it was placed in its right perspective that is in a broader considera tion of the transverse growth of the diaphysis We all know very well of course that in order to keep the same general shape while growing in length, a long bone has to distribute its transverse growth according to a pattern which changes from level to level and, at the same level from stage to stage[20, 21, 22] Let us see how Ca^{45} autoradiography exemplifies this state ment

Figure 12 7A is an autoradiograph recorded from a transverse ground section of the tibial shaft of a newborn puppy which had received Ca^{45} 24 hours before sacrifice Figure 12 7B is the microradiograph of the section itself Radioactivity is restricted to the peripheral zone and is rather evenly distributed around the shaft. In fact, the inner part of the wall, which seems unreactive has a very faint diffuse radioactivity which, for

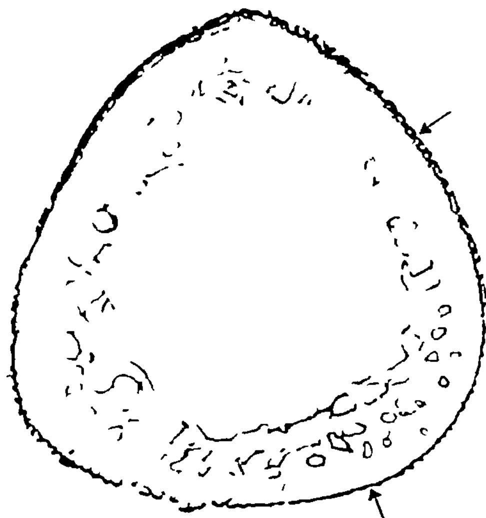


FIG. 12-8 Autoradiograph of section as in Fig 12 7*A* but from a 9-week-old dog. This illustration is reproduced at the same magnification as Fig 12 7*A* to facilitate comparison. (×17)

technical reasons fails to show up on the printed photograph. The corresponding histology (Fig 12 7C) makes the combination of the three illustrations self-explanatory Transverse growth occurs by subperiosteal formation of new trabeculae which stop growing as soon as they merge with each other Deeper in the shaft, there is no sign of osteogenesis

In the later stages of development, the picture becomes widely different [33] Figure 12-8 is an autoradiograph obtained like Fig 12 7A, but from a 9-week-old dog Both illustrations are reproduced at the same magnification to facilitate comparison. The shaft of the newborn would take place inside the older one The Haversian remodeling which had not yet begun at birth is now extremely active, even more so than in the adult (Fig 12 1) Transverse growth is obvious Where it is fast (upper arrow) its autoradiographic expression is a succession of complete or incomplete rings resulting in a chainlike appearance Where it is slow (lower arrow) the

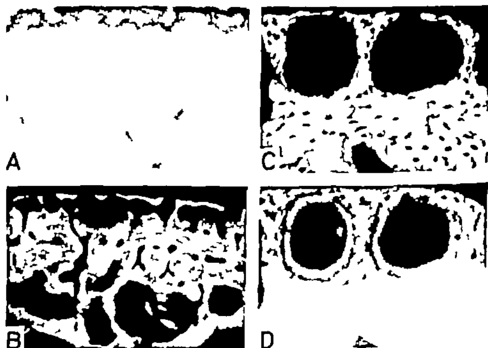


FIG 12 9 *A* Enlargement of an imprint of fast transverse growth similar to that pointed out by the upper arrow of Fig. 12-8 (×38) *B* Microradiograph of the section from which the autoradiograph shown in *A* was obtained. The thin trabeculae of bone which grow at the surface of the shaft, and which are highly radioactive are fully calcified. (×38) *C* Enlargement of a microradiograph comparable to *B* (×164) *D* Histology corresponding to *C* Growing ridges delimit grooves which are transformed afterward into tunnels The latter are subsequently filled by concentric deposition. The phenomenon begins with the deposition of a PAS-positive preosseous layer as in the Haversian remodeling (Fig 12 2A) Its final result is a row of osteons which have been added at the periphery of the shaft, unlike Haversian osteons which are substituted for preexisting bone. (×164) (*Reproduced by permission of Lea and Ponlot Arch biol Par* 69 455–465 1958)

imprint is a single line The two typical varieties of imprints may be combined or linked by intermediate aspects

A careful study of these imprints is of profit not only for my own subject but also because it leads I believe to a better understanding of "Bone as a Tissue" the general title of this Research Conference

The fast process (upper arrow of Fig 12-8) is analyzed by Fig 12 9 In A that is in the autoradiograph we notice that on the whole the chainlike design is denser than the winding lines of the Haversian remodeling In B the microradiograph corresponding to A brings its own contribution the thin trabeculae of bone which grow at the surface of the shaft, and which are the most radioactive are definitely more calcified than the lamellae being deposited in the wide and irregular cavities deep in the shaft Here again histology must supplement microradiography In C, the microradiographic aspect is seen at a higher magnification and it

Fig. 12 10 *A* Enlargement of an imprint of slow transverse grow[illegible] that pointed out by the lower arrow of Fig 12-8 (×38) *B* M[illegible] of the section from which the autoradiograph shown in *A* [illegible] The simple line of the autoradiograph corresponds to the su[illegible] bone. (×38) *C* Enlargement of a microradiograph compar[illegible] *D* Histology corresponding to *C* Black vertical arrows mark[illegible] in *C* and *D* help in observing in *D* a PAS-positive preosseous [illegible] face of the shaft This preosseous layer seems identical with the [illegible] of the Haversian remodeling (white arrow) But it calcif[illegible] by the examination of the Haversian osteon pointed out [illegible] arrow (×164) (*Reproduced by permission of Lea and* [illegible] *Par* 69 455–465 1958)

should be considered together with its histologic counterpart, in D succession of events is as follows Parallel ridges begin to appear ar come more and more salient, delimiting grooves between them. Ea the ridges assumes the shape of a T the horizontal bar of which n with those of adjoining ridges In this way the grooves are transfo into tunnels, which are subsequently filled by concentric deposition [a] final result is a row of osteons lying side by side and making the supe layer of the shaft. It had not been noticed previously that the phenon begins with the formation of a thick PAS-positive preosseous layer calcifies suddenly and heavily

The word "osteon" is currently used to mean the end product c Haversian remodeling, that is, a structure filling a cavity hollowed c preexisting bone We are now led to recognize that these "replacer osteons are by no means the only ones and that there are osteons c other kind which are added to preexisting bone, not substituted for it which might be called addition" osteons

From their mode of origin we may expect to find that the two kir osteons differ in structure at their periphery Such is indeed the ca replacement osteon is delimited by a cementing line whereas there such line between addition osteons [33]

The slow process of transverse growth (lower arrow of Fig 12- illustrated by Fig 12 10 The autoradiograph (A) is read with the a the corresponding microradiograph (B) which, in turn, is explaine C and D that is by another similar field microradiographed and obs under the microscope In C and D black vertical arrows marking the cells help to observe a PAS-positive preosseous layer which is even a surface of the shaft This preosseous layer seems identical with the p seous layer of the Haversian remodeling (white arrow) But it cal *faster this being proved by the same document there is a higher cal* content around the cells shown by the black vertical arrows than ir depositing Haversian osteon pointed out by a black oblique arrow

Currently the word osteon is also more or less synonymous with unit of structure of compact bone " *For all practical purposes the cu* may be followed But the concept is not fundamental because it does cover the product of the slow process of transverse growth, a product w has the right to claim the same citizenship as the osteons.

The late processes that we have been studying are extremely impor quantitatively in the building up of an adult skeletal piece Much of tissue that they form will persist in adult life whereas nothing (ex for very limited regions) will remain of the primary bone elaborated ir initial stages

Figure 12 11 is the microradiograph of the radius diaphysis of an a dog. It illustrates the end result of the late processes of transverse gro

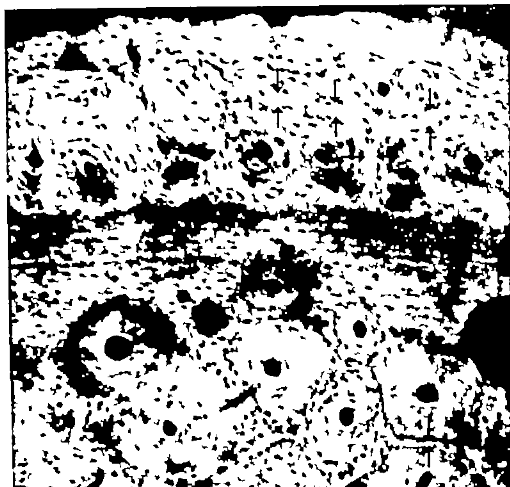


Fig 12 11 Microradiograph of a transverse section of the radius diaphysis of an adult dog, illustrating the end result of the various processes described in this chapter

The T-shaped white structure pointed out by eight arrows is not equivalent to a cementing line, such as that selected by two arrows It is the heavily calcified T-shaped bone trabecula which is seen to initiate the fast process of transverse growth in Figs. 12 9B C and D (×134)

and, in fact, the end result of the various processes described in this chapter The superficial layer has been deposited by slow transverse growth. Before that, fast transverse growth had built a row of addition osteons Still earlier the slow process was in action Deeper in the shaft, the tissue has been almost entirely renewed by the Haversian remodeling The slow and the fast processes of transverse growth may thus alternate in the same region We already know (Fig 12-8) that all possible variations are observed between their autoradiographic expressions arbitrarily selected as typical.

Figure 12 11 shows also the real cementing lines in the depth of the tissue one of them being isolated by two arrows in the lower right corner of the illustration The T-shaped white and thick boundary shown by eight

arrows around part of the addition osteons is not a cementing line it is the heavily calcified T-shaped bone trabecula which, as we have seen, initiates the fast process of transverse growth

Remarks

As stated at the beginning, the facts are looked upon from the morphologic standpoint. All the localized imprints of radioactivity are seen to correspond to calcification processes and there is a direct relationship between the density of the imprints and the rate of the subjacent calcification processes. I do not imply that everything in the Ca^{45} autoradiograph merely means calcification More particularly the diffuse reaction is not accounted for in this study

Autoradiography gives an excellent visualization of the storage of calcium in the skeleton I have suggested [36] that the regions where calcium is stored might also be those from which it is in large part released. This view seems to fit in with current concepts of bone metabolism based on other grounds [37, 38] But, strictly speaking, the decisive proof is still lacking.

Lastly I should like to believe that autoradiography may be of value in interpreting the observations made with Ca^{47} and Sr^{85} [39] Sooner or later the data recorded by the scintillation counter will have to be explained in terms of the histologic distribution of the isotope.

Summary

Ca^{45} autoradiographs of adult and growing bone are analyzed in terms of the subjacent calcification processes revealed by microradiography combined with histology

Acknowledgments

This presentation is based chiefly on unpublished research recently carried out in the author's laboratory by R. Ponlot, who will give a full account of his work elsewhere.

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DISCUSSION

Ultrastructure of Bone

Chairman Friedrich Wassermann, M.D., Ph.D

CHAIRMAN WASSERMANN Dr Fitton Jackson will start the discussion by answering the questions directed to her

DR. FITTON JACKSON I have one question here from Dr Bassett "Do the collagen fibrils near the cell membrane ever have a smaller diameter or shorter length than those some distance out in the matrix?"

In the bone there is a variety of sizes of diameter but I do not think there is any significant size gradient in this variation that I have noticed between those adjacent to the cell membrane and those farther out.

In tendon, however I can state quite distinctly that, in various ages studied in avian tendon material from about 8 days embryonic up to 6 months postembryonic (cock) the variation of diameter of the fibrils between those near the cell membrane and farther away is minimal.

From Dr Felix Bronner this question "Would you kindly summarize once again the evidence supporting the distinction between fibers and matrix?" Dr Robinson also has this question jointly Will we each define matrix? For this purpose I was trying to make a distinction between the collagenous fibers and the material surrounding them and I believe it is the old histologists of the last century mainly from Germany who distinguished the fibrous component from that which they called matrix, and it is this definition which I have followed—in other words, the matrix can be equated with the ground substance Would you not support this?

CHAIRMAN WASSERMANN I support it.

DR. FITTON JACKSON And the collagen fibers are distinct from this matrix.

Another question "*How does cortisone therapy affect fibrogenesis and matrix formation?*"

I myself have not done any work on this point and I am not too familiar with the literature but I believe one or two people have found that cortisone does inhibit the formation of the fibers, but I think it is rather an open question and not a great deal of work has been done on it.

CHAIRMAN WASSERMANN May I be permitted to make a few comments concerning the question about the size of young fibrils as compared to that of older ones by showing in a few electron micrographs some results of an investigation of fibrillogenesis in the regenerating tendon of guinea pigs which after tenotomy were recovering from experimental scorbutus

If we readminister ascorbic acid to vitamin C deficient animals whose Achilles tendons were cut, we initiate the regeneration which cannot begin

during the deficiency Thereby we are able to catch the earliest manifestations of fibrillogenesis By repeating the procedure a few times that is by allowing the tenotomized animals to recover by making them sick again and allowing them to recover after a certain time again we find in the same tissue the first stages of fibrillogenesis and in addition fibrils formed during the first recovery

(*Slide*) First I show you an inactive fibroblast from a scorbutic animal In contrast to this cell you notice in the fibroblast from a recovering animal (*slide*) a homogeneous surface layer This newly formed exoplasmic zone which is free of endoplasmic reticulum and of mitochondria, contains very fine fibrillous structures of about 50 A in width Since we often find immediately attached to this zone the youngest continuous fibrils (*slide*) I do not hesitate to interpret the fine structures in the exoplasmic zone as the plurimacromolecular precursors of the precollagenous "primary" fibrils Next (*slide*) you see, adjacent to a fibroblast which shows the beginning of activity thicker fibrils with diameters of about 500 A Since this is a preparation from an animal that was recovering for the second time the fibrils outside the cell are undoubtedly some of those produced during the first recovery By correlating such pictures with the experimental data, we hope to be able to estimate approximately the age of fibrils and to obtain data concerning the growth of the fibrils from the beginning of fibrillogenesis to the end of a second period of recovery

DR. FITTON JACKSON Yes I think it is a question of when you quite call a fibril a fibril and what size diameter you agree to In other words how many collagen molecules are banded together both longitudinally and laterally before a definite fibril with the accepted cross-striation and periodicity is obtained

DR ROBINSON I suppose that we had better take up the definitions first. There seems to be difficulty in the definition of *matrix* I have thought of matrix in terms of a section of decalcified bone that one observes in the light microscope One sees, with the usual hematoxylin and eosin stain a pink region lying between the cells in the decalcified bone tissue In this one won t usually see any fibers unless special stains are applied One sees just a pink amorphous region and the term that most of us use, I think is "matrix" for this pink-staining material

On the other hand, other people consider that matrix is just the material between the fibrils in this pink-stained area I think that Dr Fitton Jackson points out that this is the thing that she calls matrix. I call this the mucopolysaccharide on cement substance space around the fibrils.

I call the fibrils and this mucopolysaccharide space (which contains dissolved salts undoubtedly some tropocollagen units, and probably water in a high degree) the matrix

The definition of *osteoid* comes up It has been an idea that osteoid is

a separate thing from bone matrix after calcification has occurred When I began thinking about bones many people had the idea that somehow or other osteoid, which one sees prior to calcification, was removed and calcium phosphate crystals sort of took its place. I think the evidence now shows that the solids in that osteoid remain during the calcification process, but the water that was there originally disappears as the apatite crystals move in

Therefore, the term osteoid is changing its meaning. We can have osteoid that is 50 per cent calcified we can have osteoid that is 10 per cent calcified or we can have osteoid that is "fully" calcified, that is, perhaps about 90 per cent of theoretically complete calcification.

I have used the term calcified osteoid wrongly perhaps, but it does, I think, give one the idea that the material that is called osteoid doesn't change a great deal, so far as we can tell, in its composition, its solid composition, during the calcification process

A question from Dr Marshall "Do you feel that our measurements of mineral density as a function of age are consistent with yours?"

The simple answer to this is yes In other words, in bone from a very young animal there will be more regions of the bone matrix or calcified osteoid that are incompletely calcified. On the other hand, in the older bone a larger percentage of this bone matrix will be more completely or "fully" calcified. Therefore we agree that in the younger bone the general density the over all density of the matrix will be less than it is usually in the mature animal. However in that young bone probably 50 per cent will be just as calcified as in the matrix of the old bone or the bone from the older animal.

Incidentally no areas of supercalcification appear in the bone matrix as far as Marshall and Rowland and Jowsey can tell.

Dr John Royal Moore asks me, "Does the apatite crystal become amorphous at 500 C?"

So far as we can tell the crystals, the little crystals in bone at 500°C, gradually melt one into the other so that bigger crystals are produced as one goes from 500 up toward 1200 C These crystals apparently lose water that small moiety of water in the crystals during the process As the crystals grow they demonstrate a more perfect atomic lattice pattern as demonstrated by x-ray diffraction patterns

"Are crystallization and calcification used synonymously?"

I think that the little hydroxyapatite crystals fill in the matrix water space This summarizes my present notion of "calcification" of the matrix. The crystals however may form on an organic template rather than precipitate from extracellular fluid as the term "crystallization" tends to imply

"Do you not show calcification within the fibers?"

Yes we showed I believe calcification within the fibrils and I think

perhaps some of Dr Jackson s electron micrographs also show calcifica tion inside the fibril

"Please clarify Dr Frost s statement re osteoporosis"

It would be rather impertinent to try to clarify somebody else s state ment. I will just tell you what my impression of his statement is. In certain patients having osteoporosis, one not only finds less bone tissue per unit volume of a bone but there also may be areas of bone tissue which are not so fully calcified as one would expect.

Dr Freeman asks "Have you ever studied the hydration of the primitive bone found in Albers Schönberg s disease?"

No

CHAIRMAN WASSERMANN Dr Engström.

DR. ENGSTRÖM I have a question first, from Dr Costich, and another one, anonymous, about that statement I made that there is perhaps only 10 per cent difference between the black and white areas in a microradiogram "Is that actually supported by measurement?" one question asks I can say that it is supported by measurements and it is inherent in the nature of the technique When you use relatively hard x rays, as in this case you overexpose your microradiograms, you will notice that background be comes completely dark. That means that the incident x ray intensity is high and what you pick up are very small differences in mineralization You can actually by this technique of overexposure distinguish as small differences in mineral content as a few tenths of a per cent. The only statement I want to make is that one has to be careful not to let the appearance of black and white suggest to you that you have a tremendous difference in mineral content

Everyone who has worked with quantitative microradiography knows that, if you expose the microradiogram so that you can measure the inci dent x ray intensity by photographic density this means that the density corresponding to the incident beam should not exceed 1 then you don't see by eye any of these differences in the microradiogram, but you can pick them up by photometry This overexposure with relatively hard x rays therefore is a means of demonstrating these small differences which exist between the osteons, and that is why the osteoid doesn t show as Professor Lacroix has shown On the other hand you can show such structures by changing the x-ray energy

The high sensitivity of the microradiographic method relies on the fact that the fine-grain photographic emulsions when overexposed, have a high gamma value and therefore are a most efficient amplifier in themselves for small intensity differences

Then from Dr Hurley there is the question "What is the effect of heat—100 to 150°C—on the size shape and structure of bone crystal?"

Well, one can only so far describe and not exactly interpret the observed value or the pure width of some of the wide-angle reflections Cooked bone

will change the observed width by about 4 or 5 per cent, which would mean a corresponding increase in size

If you go to glycerol ashed bone for example then the width, that is, the pure width, becomes smaller consequently the crystallites are larger If you go up to 500°C, still smaller width and above 800 C the diffraction lines are as sharp as those from almost infinitely large crystals. In fact, this is the way you set your standard for profile measurements

And then there is the question from Dr Bassett "In view of your recent findings do you believe that the hydroxyapatite crystals can exist as hexagonal plaques?"

As shown by many workers and notably now by Glimcher when you try to prepare these hydroxyapatites you can get all sorts of shapes, depending upon the conditions But I want again to state that, when you look at bone with x-ray refraction methods, the inherent nature of the method is such that perhaps the technique picks out only a certain fraction which dominates in diffraction. That is perhaps why we get relatively homogeneous results, and the only correct way to define crystallites in bone would be to give a range distribution of crystallites that would account for their crystallographic properties, but we haven t any method of doing this at the moment.

Then there is the question about strontium in the diet in our animals, and in the first experiment we actually tried to replace all calcium with strontium the rats of course picked up the calcium which we couldn t remove from the diet and they developed the well-known strontium rickets and utilized that small amount of calcium In fact these rats produced off spring which we fed in the same way

The total content of strontium was, as I said, about 10 per cent as determined by x-ray fluorescence The same results have been reported by Russian workers. We have not published these results yet.

And then there is a question "What is the per cent calcium in bone?"

Well, I think one can only answer as the old fellow in the archipelago in Sweden with eight children who when the minister asked him, "What s the name of your children?" he said "It s different."

CHAIRMAN WASSERMANN Dr Lacroix.

DR LACROIX The first question is from Felix Bronner "Will osteon remodeling occur in parts of shafts that have previously been laid down in layers?"

Yes, but with some delay There is a lag. The Haversian remodelling follows in the wake of the deposition of periosteal layers In the human at the end of growth, these thick peripheral layers are conspicuous. After several years they are thinned out and replaced by substitution osteons.

The second question is "Would you kindly comment on the contribution of the periosteum to the formation of additional osteons?"

The periosteum supplies the cells. This is shown by transplantation

experiments When grafted under the kidney capsule, the periosteum is osteogenic. Other sites of transplantation are not so good for this type of experiments.

"Do you see low-density calcification on resorbing surfaces of old osteons?"

No I don t think there is such a region of low density We must be careful to avoid a possible mistake If the limits of a resorption cavity are beveled the x ray picture might suggest that there is a layer of low density at the periphery of the cavity

"In incompletely calcified osteons is matrix fully laid down? What is the relation of osteoblasts and osteocytes in incompletely calcified osteons?"

I think that any answer to the first part of the question "Is matrix fully laid down?" would be purely semantic. As far as the relation of osteoblasts and osteocytes is concerned, there is a continuity of the canaliculi going from the preosseous layer to the fully calcified bone Such a continuity is distinctly seen with Sudan black B Most likely the osteoblasts covering the inner surface of the preosseous layer send off processes which are progres sively embedded in the newly deposited matrix

Dr Bachra asks, "Would you be in a position to comment on the transformation of the orthochromatic layer into a metachromatic one and its relationship to calcifiability?"

This is the main problem I have shown what I had seen without going into too much interpretation. It looks as if calcifiability requires a critical configuration between collagen and mucopolysaccharide At any rate the preosseous layer which is deposited must undergo some unknown transformation before it is able to calcify

The last two questions are of a technical nature and they might be linked together "How do you prepare your slides for microradiography? What methods do you use to prepare sections of bone?"

The methods are rather simple Compact bone from large animals is not embedded Compact bone from small animals or cancellous bone is always embedded in methylmethacrylate The bone or the block is cut with a diamond saw The sections are not thin enough and they are ground first with abrasive papers, and then with ground glass The section is microradiographed A contact autoradiograph is then recorded After that, the section is examined histologically Of course cytologic details are lost but the histologic pictures are still useful as you have seen

CHAIRMAN WASSERMANN In the name of the panel members, I want to thank Dr Nicholson and his co-workers for the excellent organization of this fine section which led us from submicroscopic processes over the physical chemical state of the matrix and the size of the crystals, finally to the bone as a whole

Part IV

Vitamin D, Parathyroids, Citric Acid, Calcium, and Phosphorus

Chairman

John Eager Howard, M D

Department of Medicine
The Johns Hopkins Hospital Baltimore

13

Parathyroids and Homeostasis of Blood Calcium*

D Harold Copp, M.D , Ph.D

Department of Physiology University of British Columbia
Vancouver Canada

Although less than 0 1 per cent of the total calcium in the body is present in the blood and extracellular fluid, this calcium has a very vital role in the function of the body affecting enzyme activity membrane permeability and neuromuscular excitability The level of blood calcium has been described by McLean and Hastings as one of Nature s physiological constants " [2] and indeed it is perhaps the best regulated ion in blood In 414 determinations of plasma calcium in 96 normal fasted dogs the average was 9 89 ± 0.54 mg per 100 cc (standard deviation) with a standard error of less than 0.3 per cent. In 196 determinations in 27 parathyroidectomized dogs the average was 5 84 ± 1 15 mg per 100 cc (standard deviation) with a standard error of 1 4 per cent. The values were obtained by photometric titration with ethylenediaminetetraacetate (EDTA) using a modification of the method of Lehmann.[3] The frequency distribution of these values is shown in Fig 13 1 The constancy of blood calcium is maintained by the addition of variable amounts of calcium absorbed from the intestinal tract or released by bone resorption and by the removal of calcium by excretion in urine and feces and by deposition in bone

Although dietary intake and excretion may affect the net calcium balance the homeostasis of blood calcium appears to depend primarily on bone and parathyroid glands This has been discussed in a number of recent reviews [4 7] MacCallum and Voegtlin observed in 1909 that the serum calcium level falls after parathyroidectomy [8] Typical curves in an adult dog and a young 2 month-old pig are shown in Fig 13-2 The extremely rapid bone growth in the latter is associated with a precipitous drop

* Aided by grants from the division of Medical Research National Research Council of Canada.

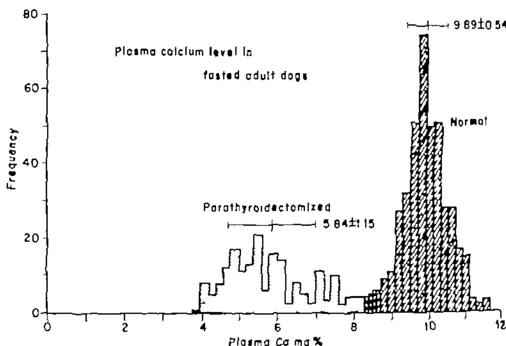


FIG 13-1 Frequency distribution of plasma calcium values in normal and parathyroidectomized dogs

Parathyroidectomy
Normal range
Adult dog
Pig – 9 weeks
Plasma Ca mg %
10
5
0
0
10
20
30
Time in hours

FIG. 13 2. Fall in plasma calcium following parathyroidectomy in an adult dog and a young 9-week-old pig.

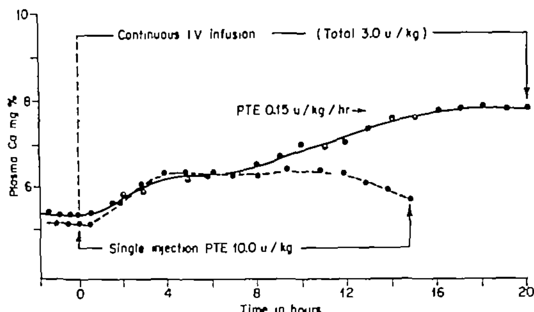


FIG. 13 3 Effect of a single injection of 10 units parathyroid extract per kilogram and a continuous intravenous infusion of 0 15 units parathyroid extract per kilogram per hour in a parathyroidectomized dog.

in the plasma calcium level, but in both cases this levels off at about 50 to 60 per cent of the normal value Talmage and Elliott have shown by peritoneal lavage that parathyroidectomized rats can mobilize calcium from bone to maintain this level at almost the same rate as normal rats.[9]

The effect of intravenous infusion of parathyroid extract† was studied

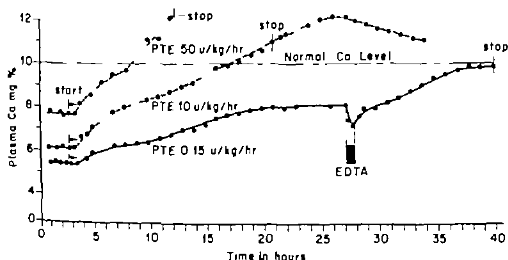


FIG 13-4 Effect of continuous intravenous infusion of parathyroid extract at various dose levels in parathyroidectomized dogs.

† The parathyroid extract used was supplied through the courtesy of the Eli Lilly Company of Indianapolis.

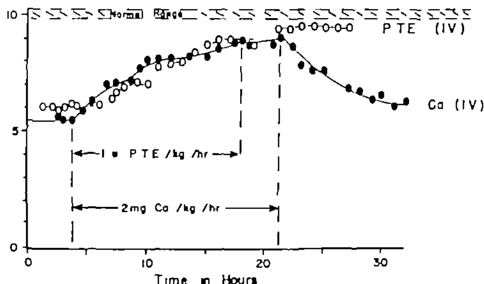


FIG 13 5 Comparison of the effect of continuous intravenous infusion of 1 unit parathyroid extract per kilogram per hour with the effect of continuous intravenous infusion of 2 mg/kg/hour of calcium as calcium gluconate in the same dog

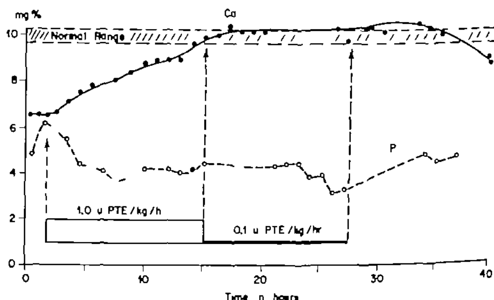


FIG. 13-6 Restoration of the normal plasma calcium level in the parathyroidectomized dog by continuous intravenous infusion of 1 unit/kg/hour and maintenance with 0 1 unit/kg/hour

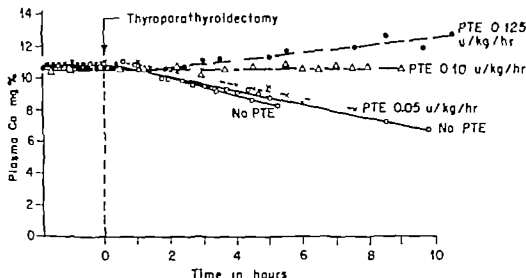


FIG. 13-7 Maintenance of the plasma calcium level following parathyroidectomy Effect of various dose levels of parathyroid extract given by continuous intravenous infusion.

in parathyroidectomized dogs and pigs As seen in Fig. 13 3 a single injection of 10 units/kg produced a rise of approximately 1 mg per 100 cc which persisted for 10 hours Continuous infusion of a much smaller amount (0 15 unit/kg/hour) resulted in a slow and steady rise in the plasma calcium level The effects of continuous infusion at various dose

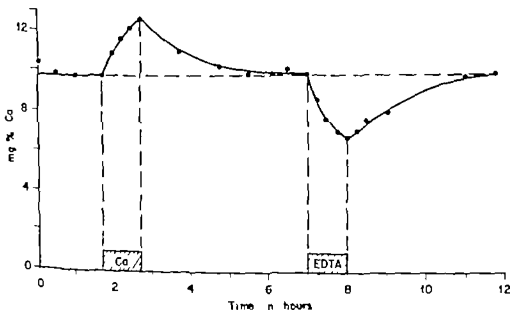


FIG. 13-8 Acute homeostasis of plasma calcium in the dog. Effect of addition of 10 mg/kg over a 1-hour period by infusion of calcium gluconate, and of removal of an equivalent amount by administration of sodium EDTA.

Fig. 13 9 Regulation of plasma calcium. Effect of addition of 10 mg of calcium per kilogram over 1 hour and removal of an equivalent amount, when the plasma calcium level is not restored to normal

levels from 0 15 to 5 units/kg/hour are shown in Fig 13-4 It will be noted that in each case there is a latent period of 20 to 40 minutes before any significant rise in the plasma calcium level is observed and the effect also persists for several hours after the infusion of the parathyroid extract has

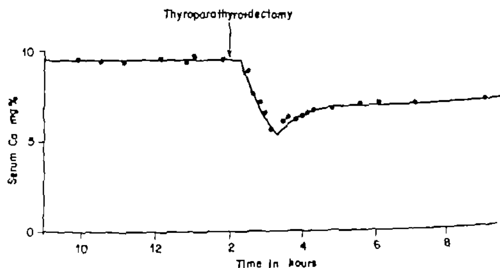


Fig. 13 10 Effect of removal of 10 mg Ca per kilogram immediately following parathyroidectomy

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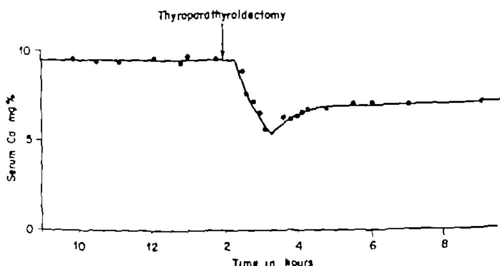


FIG. 13 10 Effect of removal of 10 mg Ca per kilogram immediately following parathyroidectomy

been discontinued This is in contrast to the effect of direct infusion of calcium gluconate as shown in Fig 13 5 In this case the blood calcium rises immediately and drops as soon as the infusion is discontinued This evidence suggests that the parathyroid extract acts by stimulating a secondary mechanism in bone, possibly by increasing some cellular activity as was suggested by Collip et al[10] It was found that continuous infusion of 1 unit/kg/hour to a parathyroidectomized dog would restore the normal plasma calcium level in 10 to 14 hours, while 0 1 unit/kg/hour was sufficient to maintain this level, as shown in Fig 13-6 It was also found that 0 1 unit/kg/hour was sufficient to maintain a normal blood calcium level for many hours if the infusion was started immediately after the surgical removal of the parathyroids from the dog as shown in Fig 13-7

Although parathyroid activity may determine the level of plasma calcium its regulation is primarily dependent on bone Hastings and Huggins[11] first demonstrated the speed with which calcium could be mobilized from bone to restore the normal blood level when it had been lowered by repeated transfusions of decalcified blood We have studied the acute homeostatic control in more than 50 dogs in which the plasma calcium was raised by intravenous infusion of calcium gluconate or lowered by the administration of isotonic sodium EDTA. The amount given was sufficient to add or remove 10 mg of calcium per kilogram body weight in a 1-hour period In over half of the normal animals, blood levels were restored within 4 to 6 hours, as shown in Fig 13-8 However in some dogs the blood level remained high after calcium infusion or remained at a subnormal level after EDTA This is shown in Fig 13-9 This was also observed when the EDTA infusion was given shortly after parathyroidectomy as shown in Fig 13 10 Apparently functional parathyroid glands are necessary for restoration of the normal level.

Calcium gluconate was infused into parathyroidectomized dogs several days or weeks after the operation when the plasma calcium had stabilized

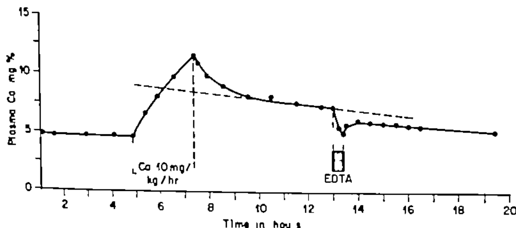


FIG. 13-11 Storage of calcium in the parathyroidectomized dog

FIG. 13-9 Regulation of plasma calcium. Effect of addition of 10 mg of calcium per kilogram over 1 hour and removal of an equivalent amount, when the plasma calcium level is not restored to normal.

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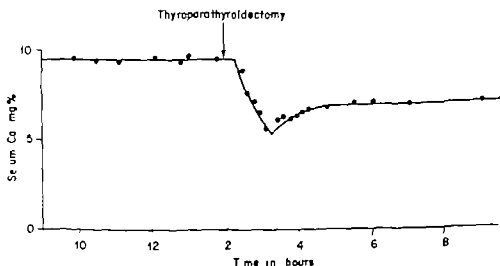


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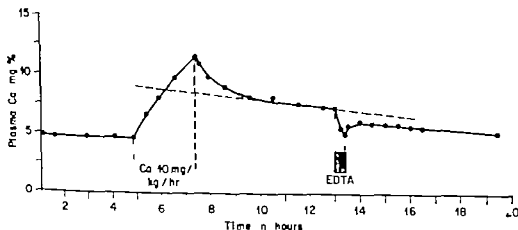


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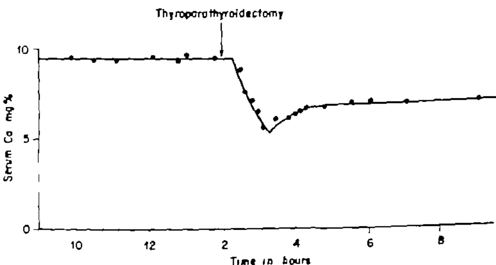


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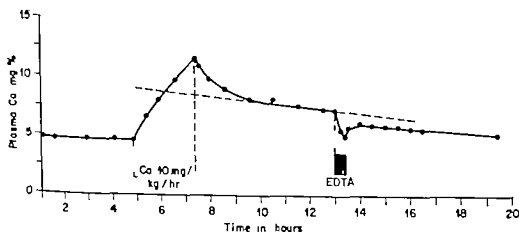


FIG 13-11 Storage of calcium in the parathyroidectomized dog.

at the new low level. In all cases, the plasma calcium did not return immediately to the preinjection level but remained elevated. This is shown in Fig 13-11 Since only a limited amount of calcium can be accounted for by the increased Ca in extracellular fluid, it is suggested that the balance has been stored in a labile pool in bone which is in equilibrium with the plasma calcium A model of this hypothetical system is shown in Fig 13-12. Because of the rapid interchange between plasma calcium and that in interstitial fluid,[12] calcium pools can be considered a single compartment with a dimension of approximately 15 mg/kg body weight. Analysis of our curves indicates that the hypothetical calcium storage pool in equilibrium with plasma ranged from 30 to 80 mg/kg in the dogs studied. Its dimension is similar to that of the exchangeable bone calcium, as measured with radiocalcium.[18] This labile calcium pool in bone would act as a buffer to assist in the regulation of the plasma calcium level, storing excess calcium when the blood level has been raised and providing calcium to restore the blood level after EDTA has been given Its availability is dependent on the bone

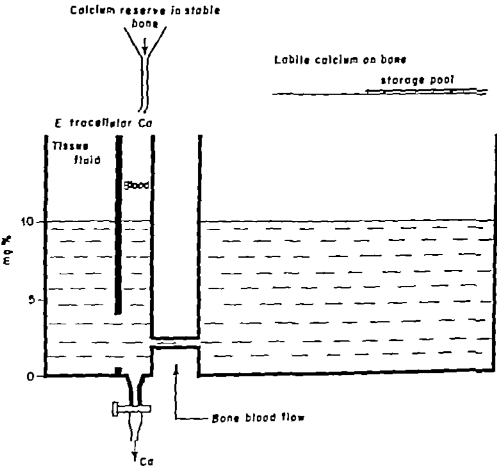


FIG 13 12. Hypothetical model of the extracellular calcium and the labile calcium storage pool in bone

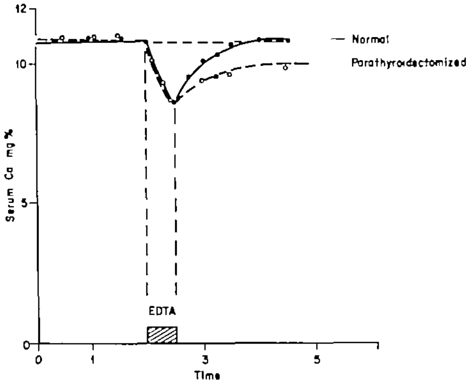


FIG. 13 13 Superimposed curves of the recovery of plasma calcium before and after parathyroidectomy in the same dog.

blood flow which from analysis of our curves appears to be 5 to 10 per cent of the resting cardiac output

In some animals restoration of the plasma calcium level after EDTA was measured, the parathyroid glands were then removed, and 4 hours later the EDTA infusion was repeated The curves obtained in the same

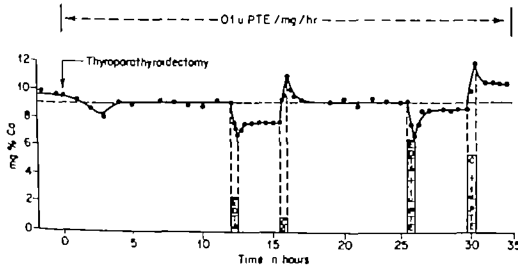


FIG. 13 14 Calcium mobilization and storage in a parathyroidectomized dog maintained on 0 1 unit parathyroid extract per kilogram per hour

animal with and without the parathyroid glands have been superimposed in Fig. 13 13 It is evident that, after the first hour mobilization of calcium from bone is greater when the parathyroids are present, and it is suggested that the period of hypocalcemia has stimulated increased output of hormone to make this possible. An experiment illustrating this effect is shown in Fig 13-14 A fasted dog was thyroparathyroidectomized and was then given a continuous intravenous infusion of 0 1 unit/kg/hour for the duration of the experiment to maintain a constant blood level. When EDTA was given alone, there was a persistent lowering of the plasma calcium level (and presumably of the hypothetical labile bone pool) suggesting that no added mobilization of calcium had occurred When an equivalent amount of calcium was added, both levels were restored to normal. However when 1 unit of parathyroid extract per kilogram was given at the same time as EDTA, additional mobilization of calcium did occur which in this animal has been estimated at 7 mg/kg Addition of a similar amount of parathyroid extract to the calcium infusion resulted in a further mobilization of calcium of about the same magnitude

Summary and Conclusions

It is now possible to speculate about the mechanisms responsible for the very precise regulation of the plasma calcium level. The main factors involved are shown diagrammatically in Fig. 13-15 The plasma calcium exists in equilibrium with that in interstitial fluid and receives calcium from in-

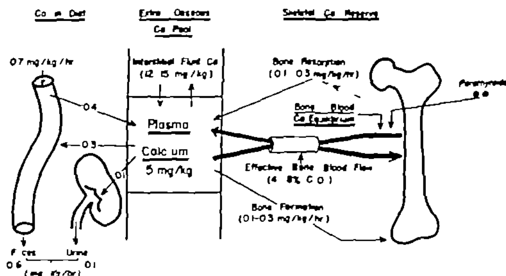


Fig 13-15 Diagram of the factors involved in regulation of plasma calcium level.

testinal absorption and from bone resorption Calcium is lost from the plasma by excretion in urine and feces and by deposition in bone Possible changes in plasma calcium level are "buffered" by the labile storage pool in bone, which may correspond to calcium in the hydration layer and surfaces of the crystals of bone salt However maintenance of a normal level of plasma calcium depends on the activity of the parathyroid glands, which probably act by a "feed back" mechanism as suggested by McLean[5] Hypocalcemia apparently stimulates the parathyroid glands which in turn promote mobilization of calcium from bone It is probable that hypercalcemia inhibits the parathyroids. There is considerable evidence that parathyroid extract increases the activity of bone cells particularly with respect to citrate.[14, 15] Neuman et al[16] suggest that this increases the solubility of bone salt and thus promotes calcium mobilization. It may also increase the activity of calcium in the labile bone storage pool

Because the parathyroidectomized animal can mobilize calcium from its skeleton and can maintain the blood calcium even though at a level much below normal it seems likely that the hormone acts to increase the activity of a mechanism already present. In this respect it would be analogous to the thyroid hormone which is not essential for metabolic activity but is required to increase metabolism to its normal optimal level

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14

Physiology of Vitamin D*

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The mode of action of vitamin D at the cellular level has not yet been uncovered At the moment, a variety of biochemical effects of vitamin D can be described and some attempt can be made to trace their interrelationship and possible physiologic significance

Vitamin D deficiency has usually been equated with rickets For this reason it has frequently been said that vitamin D is not an essential nutrient for the albino rat since this animal does not develop rickets following simple deprivation of vitamin D In order to produce rickets in the rat, an additional distortion of the diet is needed so that the availability of phosphorus or of calcium is reduced Nevertheless, it can be shown that vitamin D deficiency per se without abnormalities of the calcium and phosphorus content of the diet does produce biochemical abnormalities in the rat which are corrected by administration of vitamin D [1]

Figure 14-1 shows the results of such experiments. Weanling rats were placed on a diet of normal mineral composition but lacking in vitamin D At the end of 3 weeks, one group of rats was bled and bone was taken for analysis and histologic examination The remaining animals were given a single dose of 100 units of vitamin D and groups were sacrificed at 24 hours, 72 hours, and 8 days following the administration of vitamin D The curves illustrating the average values for serum citrate, calcium, and phosphorus and bone citrate of the various groups of rats are shown In each instance the initial point represents the average concentrations in vitamin D deficient rats The horizontal lines mark the limits of normal values in rats given the same diet with vitamin D The vitamin D deficient rats show

* The investigations from this laboratory reported in this paper were supported by grants from the National Institutes of Health.

hypocitricemia and hypocalcemia without reduction of serum phosphorus concentrations The concentration of bone citrate is also low although histologically the bones show no evidence of rickets Following 100 units of vitamin D the serum citrate and calcium concentrations rise rapidly reaching a maximum at 3 days The serum citrate concentrations at 3 days are well above the normal range and then drop within normal limits by 8 days. The bone citrate concentrations rise with the serum citrate levels but

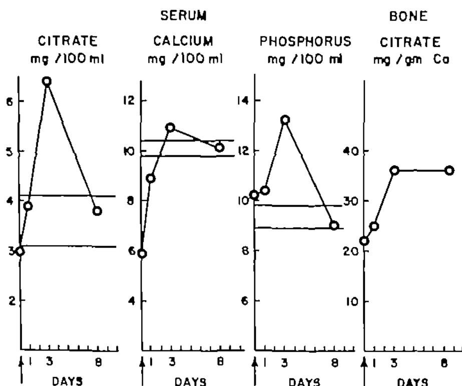


FIG 14-1 Effect of vitamin D deficiency in rats fed vitamin D free diet of normal mineral composition Initial point of each curve represents determinations in group of vitamin D deficient rats. The other points indicate the results of determinations made at 1 3 and 8 days following feeding of 100 units of vitamin D to vitamin D deficient animals. The horizontal lines mark the limits of normal serum values in rats fed the same diet supplemented with vitamin D

do not go above normal and remain constant after the 3-day interval The serum phosphorus concentrations are at the upper limits of normal and rise even higher after vitamin D is given before returning to the normal range

Although the bones show no evidences of rickets, the growth of the vitamin D deprived rats is reduced, and the bone weight is less than that of the controls so that actual total bone calcium mass is reduced This of course would be expected since absorption of dietary calcium is reduced in the vitamin D deficient animal [2] A vitamin D deficiency state can, therefore exist in the rat without true rickets The defect of calcium homeostasis shown by the vitamin D deficient animal resembles that of the hypopara

thyroid subject, and the injection of parathyroid extract into these animals is indeed without effect, unless the rats are first primed with vitamin D This suggests that the specific action of parathyroid hormone which results in mobilization of bone calcium is dependent on the presence of vitamin D or some prior action of vitamin D on the target cell This might explain the hypercitricemia seen at 72 hours, since it is probable that the parathyroid glands of these rats are hyperplastic Administration of vitamin D could then permit the excess of circulating parathyroid hormone to produce an effect on citrate metabolism which had previously been blocked by vitamin D deficit. It is known that one of the early effects of parathyroid extract is to increase serum citrate levels The action of vitamin D in mobilization of calcium from bone which has been stressed by Carlsson[3] might then be explained as an effect of parathyroid hormone which requires vitamin D for its expression.

An important difference between the response of man and the rat to vitamin D deprivation is the fact that the former tends to develop a hypophosphatemia whereas the latter as seen above maintains high serum phosphorus levels The normal or high serum phosphorus levels in the vitamin D deficient rat presumably explain the continued deposition of bone salt in the osteoid and the lack of histologic rickets The hypophosphatemia of the vitamin D deficient infant or of the osteomalacic adult is in large part the result of reduced renal tubular reabsorption of phosphate, so that it can be considered to be a form of renal hypophosphatemia The rat does not show this reaction, and renal conservation of phosphate in this species is adequate in the absence of vitamin D

Another difference between man and the rat with respect to response to vitamin D deficiency is in the occurrence of renal aminoaciduria. A generalized aminoaciduria is seen in infants with vitamin D deficiency[4] This aminoaciduria may be quite marked and is not associated with an increase in plasma amino acid concentrations, so that it can be presumed to result from impaired renal tubular reabsorption of amino acids and is another manifestation of disturbed renal tubular function The pattern of aminoaciduria is similar to that found in renal tubular injury due to lead or other heavy metals[5] or as the result of congenital metabolic disorders such as cystinosis and Wilson s disease Figure 14-2 shows a comparison of the pattern of aminoaciduria due to vitamin D deficiency rickets and that due to lead poisoning. If renal aminoaciduria is assumed to be a manifestation of renal tubular dysfunction, vitamin D deficiency in man results in functional renal tubular injury The vitamin D deficient rat on the other hand develops neither aminoaciduria nor a physiologically significant defect of renal tubular reabsorption of phosphate so that renal tubular function in this species is not obviously altered by vitamin D deficit.

Further evidence of the interrelation of renal tubular reabsorption of phosphate and amino acids and vitamin D activity is shown in studies of

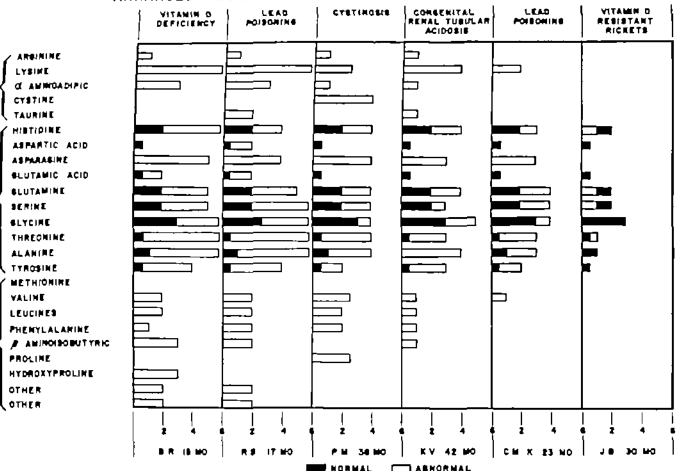


FIG 14-2. Comparison of pattern of aminoaciduria in infant with vitamin D deficiency rickets and child with lead poisoning The individual amino acid is indicated in the left hand margin and the amount of the amino acid excreted in the urine during a 24-hour period is indicated by the total length of the horizontal bar The amounts excreted by normal children are shown by the solid portion of the bar

patients with congenital renal tubular acidosis. These children with an inborn error in the mechanism for acidification of the urine develop chronic hyperchloremic acidosis followed by hypophosphatemia and rickets.[9] The hypophosphatemia which can be shown to be due to diminished tubular reabsorption of phosphate does not respond to vitamin D so long as the acidosis is uncorrected Figure 14-3 shows the concentrations of serum phosphorus, CO_2 and citrate in one such patient. Despite previous administration of vitamin D the serum phosphorus was reduced below 4 mg per 100 cc When the acidosis was corrected by administration of sodium and potassium citrate, the serum phosphorus rose sharply without further vitamin D and healing of the rickets resulted. Table 14-1 shows similar data in another Studies in three such patients indicate that acidosis blocks the action of vitamin D on tubular reabsorption of phosphate In addition a generalized aminoaciduria appears during the period of chronic

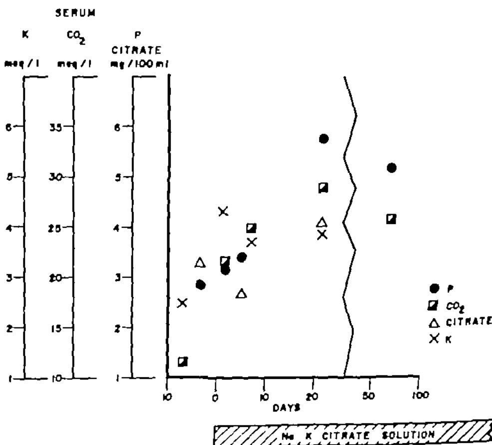


FIG 14-3 Concentrations of serum phosphorus bicarbonate and calcium of child with renal tubular acidosis as influenced by treatment. Vitamin D had been given without restoration of normal serum phosphorus levels so long as the acidosis persisted. Correction of the acidosis by oral intake of sodium potassium citrate was associated with a prompt rise of serum phosphorus concentrations and subsequent healing of the rickets.

acidosis, despite vitamin D therapy which progressively diminishes following correction of the acidosis (Fig 14-4)

The data cited above indicate that the mechanisms for renal tubular transport of phosphate and of amino acids in man require vitamin D for maximum efficiency but this concept does not explain the action of vitamin D in large doses in the hypoparathyroid subject As is well known large doses of vitamin D of the order of 2 5 to 12.5 mg (100 000 to 500 000 units) per day will not only raise the serum calcium of hypoparathyroid subjects but will lower the serum phosphorus levels to concentrations approaching the normal range This would appear to contradict the postula

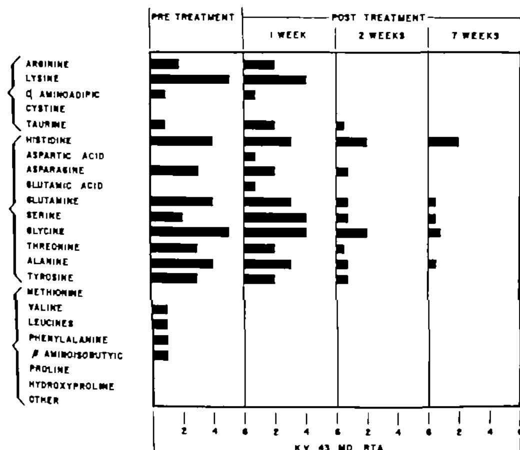


Fig 14-4 Pattern of aminoaciduria in child with congenital renal tubular acidosis. The 24-hour excretion of the individual amino acids in the urine is indicated by the length of the horizontal bars. In the pretreatment specimen a generalized aminoaciduria of significant proportions is found despite vitamin D therapy Following treatment of the acidosis with sodium potassium citrate solution, the aminoaciduria rapidly diminishes with a normal pattern after 2 weeks of treatment. The disappearance of aminoaciduria in this instance follows closely upon the return to normal of serum phosphorus levels

Table 14-1

EFFECT OF TREATMENT ON SERUM PHOSPHORUS LEVELS OF CHILD WITH CONGENITAL RENAL TUBULAR ACIDOSIS

Date	*Mg per 100 ml*		Serum phosphatase units	*mEq per liter*		
	P	Ca		CO_2	Cl	K
July 8	3 0	9.2	33.5	16.6	122	2.8
Sodium Potassium Citrate Solution						
July 23	5.7	9.8	20 1	30.8	112	4 7
July 25	6.0	0 6	24.5			
Aug. 15	6.5	9.3	15.6	30 0	108	4.[illegible]
Sept. 20	6.8	10.2	18 7			

tion that vitamin D increases renal tubular transport of phosphorus, unless it can be shown that vitamin D is required in microgram amounts for normal function of the transport system for phosphate whereas in milligram amounts it depresses renal tubular reabsorption of phosphate One possible suggestion is that vitamin D combines with the same grouping in the target organ as parathyroid hormone and that in the renal tubule cell this results in blocking of the parathyroid effect on tubular reabsorption of phosphate. Large doses of vitamin D on the other hand in the absence of parathyroid hormone may produce a parathyroid hormone-like effect. Since hypocalcemia stimulates parathyroid activity it is also probable that there is an increase of circulating parathyroid hormone in vitamin D deficiency states.

Brief mention should be made of that form of rickets in which healing may be induced without adequate correction of the hypophosphatemia I refer to vitamin D resistant rickets of the form which is usually familial and transmitted by a dominant gene [7] Sporadic cases without familial incidence are also found, including some with onset at puberty or later These patients show retarded growth, hypophosphatemia, and chronic rickets or osteomalacia although there are no other recognizable metabolic disorders. Aside from reduced renal tubular reabsorption of phosphate, no anomalies of renal tubular function are apparent. The patients whom we have studied have not shown aminoaciduria in contradistinction to infants with true vitamin D deficiency rickets, or patients with renal tubular injury resulting in the Fanconi syndrome

Treatment of these children with large doses of vitamin D of the order of several milligrams per day may produce roentgenographic evidence of healing of rickets and some acceleration of growth Even with treatment the growth rate is usually not beyond the low normal range. The serum phosphorus values remain low although some irregular and variable re

sponse is seen and studies indicate that tubular reabsorption of phosphate is persistently reduced Healing of rickets in these children may chiefly be the result of increased absorption of calcium and phosphorus from the intestine so that the concentrations of these ions may rise temporarily following a meal permitting initiation of the crystallization of bone salt in the

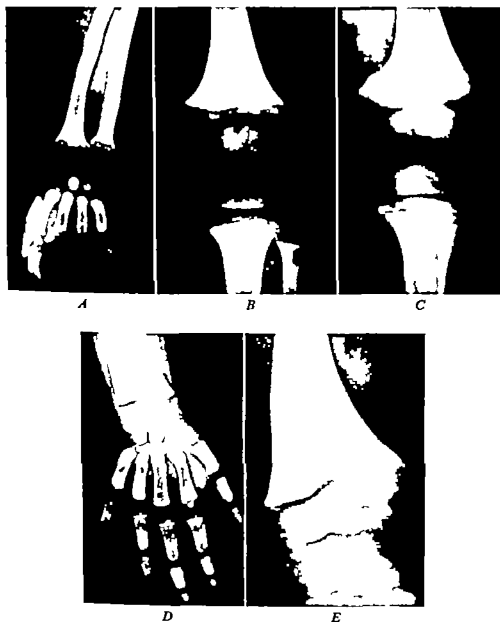


FIG. 14-5 Vitamin D resistant rickets, familial type G M age 18 months at start of therapy The series of bone roentgenograms indicate the progressive healing of active rickets when dihydrotachysterol (Hytakerol) was given as the only form of therapy *A* July 31 1959 wrist, active rickets—start of therapy *B* July 31 1957 knee active rickets. *C* Sept. 11 1957 knee, healing rickets, Hytakerol treatment. *D* Sept. 16 1958 wrist, healed rickets, 12 months after onset of treatment. *E* July 16 1958 knee healed rickets

matrix and then further deposition of bone salt around this nucleus. Yendt and Howard[8] have suggested a similar mechanism as the basis for the healing of rickets observed in rats following oral intake of citrate Initiation of healing of rickets in these patients can also be produced by repeated intravenous injections of phosphate which produce intermittent elevations of the concentrations of inorganic phosphate in extracellular fluid[9] The healing of rickets without sustained elevation of extracellular phosphate is, however usually incomplete and recurrence of active rickets may result.

One of the interesting features of this type of rickets is the fact that a preparation of dihydrotachysterol (DHT) which has exceedingly little antirachitic activity in the vitamin D deficient rat[10] is at least equipotent with vitamin D in the treatment of vitamin D resistant rickets. In Fig 14-5 a series of bone x rays in an 18-month-old child with the familial form of this disorder are shown Treatment with Hytakerol (the commercial preparation of dihydrotachysterol) in a dose equivalent to 1 25 mg of dihydrotachysterol per day produced excellent healing of the rickets. The data in Table 14-2 show that this treatment was accompanied by some in-

Table 14-2

EFFECT OF TREATMENT ON RESISTANT RICKETS

Age months	*Mg per 100 ml* Ca	Serum P	*Phosphatase units*	*Treatment*
18	10.2	2 1	34.9	DHT 2.5 mg
20	10 1	3.2	32.3	
23	10 8	4 3	22.2	DHT 1.25 mg
24				DHT 0 025 mg
28	9 4	3 7	13.3	
32	9 9	3.7	8.8	

crease of serum phosphorus concentrations, but normal values for this age were not attained X rays of a 3 year-old child with vitamin D resistant rickets without familial incidence (Fig 14-6) indicated that slow but progressive healing resulted when 2.5 mg of dihydrotachysterol was given daily This child had shown no response to a single dose of 15 mg of vitamin D and the serum phosphorus levels remained low during the period of treatment (Table 14-3)

The response of these patients to a sterol which cannot replace vitamin D in the vitamin D deprived subject raises questions as to the molecular specificity required for vitamin D activity and the basis for the refractoriness of these patients to vitamin D As yet this last question is unanswered but the behavior of these patients suggests that there may be two distinct types of action of vitamin D one which requires a very specific molecular

configuration and which is accomplished by extremely small amounts of the specific sterol and a second less specific action which is effected by relatively large amounts of vitamin D but also by other closely related sterols The first type of action is lacking in patients with vitamin D resistant rickets, whereas the second occurs and can in part substitute for

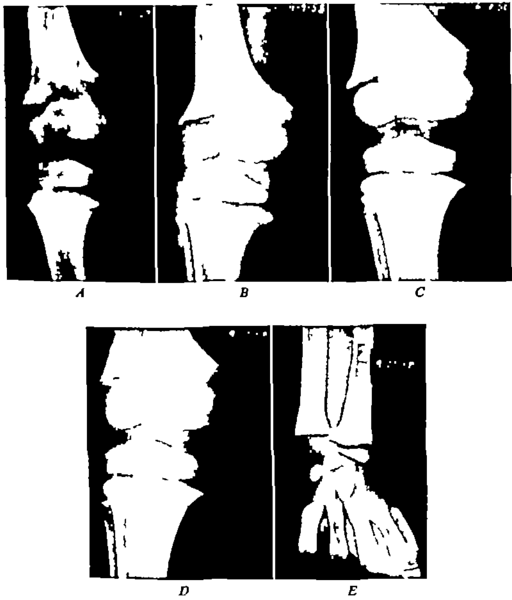


FIG. 14-6 Vitamin D resistant rickets no familial history B H age 3 years The series of bone roentgenograms indicates progressive healing of rickets when 2.5 mg of dihydrotachysterol was given daily as the sole form of therapy *A* Nov 11 1957 knee active rickets *B* Apr 9 1958 knee, partial healing of rickets following 4 months of dihydrotachysterol treatment. *C* June 10 1958 knee continued healing on dihydrotachysterol treatment. *D* Sept. 17 1958 knee almost complete healing following 9 months of treatment *E* Sept. 17 1958 wrist no signs of active rickets

Table 14-3

EFFECT OF TREATMENT ON RESISTANT RICKETS

Date	Mg per 100 ml Ca	Serum P	*Citrate*	*Phosphatase units*	*Treatment*
Dec. 6, 1957	10 8	3 1	2.8	20.8	D_2 15 mg
Dec. 12	10.5	2.5	2.9	31 9	
Dec 18	9 7	2.8		25.2	DHT 2.5 mg/day
Feb 5 1958	9 9	2 7		33 3	
Apr 2	10 6	3 0	3.3	30 1	
July 18	10 7	3.2	2.0	20 4	
Sept 17	9.8	2.8		20.5	

the more specific effect of vitamin D in so far as intestinal absorption of calcium and phosphorus is concerned.

Although the action of vitamin D at the cellular level is unsolved, a number of observations during the past few years have offered new possibilities of an answer to this problem. I refer particularly to those studies which indicate an effect of vitamin D on citrate metabolism. The action of vitamin D in increasing serum and bone citrate concentrations in the vitamin D deficient rat has already been referred to and a similar effect of vitamin D on the serum citrate levels of the rachitic infant has also been found [11] One of the theories developed from such observations is that vitamin D influences the rate of citrate accumulation in cells possibly by reducing its conversion to α-ketoglutarate DeLuca, Steenbock, and their colleagues[12-14] in a series of papers have reported an effect of vitamin D upon tissue citrate concentrations and have also found that the utilization of citrate in vitro by rat kidney homogenates and mitochondrial preparations is less in preparations obtained from vitamin D treated rats than in those from vitamin D deficient animals An effect of addition of vitamin D in vitro upon citrate utilization by rat kidney has also been found. Studies of the enzymatic activity of cartilage by Gutman[15] and of bone by Dixon and Perkins[16] have demonstrated a number of enzyme systems concerned with the glycogenolytic cycle and the citric acid cycle The presence of citrogenase in bone was demonstrated by Dixon and Perkins and more recent studies have shown that isocitric dehydrogenase activity is also present.[17] Possible differences between the cartilage of rachitic and vitamin D treated animals with respect to the activity of citrogenase[18] or the utilization of pyruvate have been suggested.[19] Nevertheless no definite evidence of inter action of vitamin D in a specific enzyme system has been established, and certain of the effects ascribed to vitamin D might be indirect since citrate utilization in vitro is also affected by the concentrations of calcium and inorganic phosphate in the medium.

Our own studies have led to another possible suggestion namely that the effect of vitamin D is upon the distribution of citrate between cells and extracellular fluid rather than upon intracellular accumulation of citrate The significance of changes in serum citrate concentration following vitamin D or parathyroid hormone as an essential factor in their effects upon the movement of calcium or phosphate ions is thrown into doubt by experiments with adrenocortical steroids Cortisol reduces serum citrate levels in the rachitic rat and blocks the vitamin D effect upon serum and bone citrate

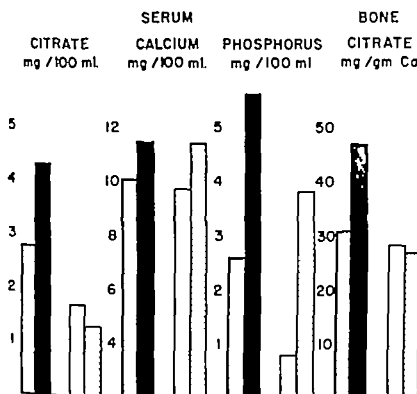


FIG. 14-7 Effect of cortisol on concentrations of citrate in serum and bone and on concentration of calcium in serum in relation to the action of vitamin D In each set of four columns the first pair of columns indicates the results in control rats not given cortisol, and the second pair the findings in rats treated with cortisol. The first column of each pair gives the average value before vitamin D treatment and the second column the average following 100 units of vitamin D

concentrations (Fig. 14-7) Nevertheless cortisol does not prevent the effect of vitamin D in raising serum calcium levels and does not block its antirachitic action[20] nor the toxic effect of large amounts of vitamin D[21] Studies of the effect of cortisol on citrate metabolism by means of tissue analyses indicate that cortisol alters the distribution of citrate between cells and serum rather than the cellular accumulation of citrate For example in fluoroacetate injected rats treatment with cortisol does not alter the intracellular accumulation of citrate but does prevent the concentration of

extracellular citrate from rising as rapidly as in control rats, so that distribution ratios of citrate between cells and extracellular phase are increased (Fig 14-8) Such experiments suggest that cortisol reduces the rate of transfer of citrate across the cell membrane

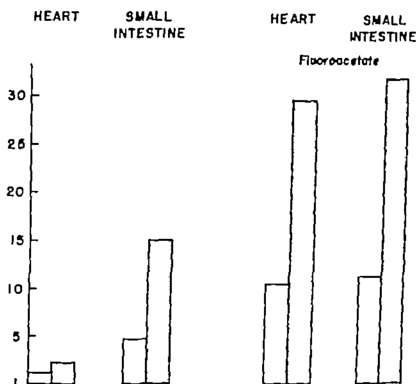


FIG. 14-8 Distribution ratios of citrate between cellular fluid and extracellular phase in tissues of control and fluoroacetate injected rats with and without cortisol treatment. The hollow column gives the value in tissues of non-cortisol treated rats and the stippled column the value in tissues of cortisol treated animals. The effect of cortisol is to increase the distribution ratio since the concentration of citrate in extracellular fluid is reduced without reduction of the concentration of tissue citrate.

Since cortisol blocks the citrate-increasing action of vitamin D without preventing its essential antirachitic activity the citrate effect does not appear to be the primary biochemical basis of vitamin D action Nevertheless, the citrate phenomenon may be a clue to the nature of vitamin D action since the movement of citrate is possibly related to the transport of ions across cell membranes Vitamin D does apparently influence the rate of movement of calcium and phosphate ions across the intestinal mucosa and renal tubular cells and the movement of calcium between the solid phase of bone salt and body fluids In certain of these functions, an interaction of vitamin D and parathyroid hormone seems to exist Elucidation of vitamin D action and explanation of parathyroid hormone effect are probably mutually dependent.

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15

Citrate Metabolism with Special Reference to Calcium and Bone

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Without the aid of some driving force normally mediated by the para thyroid glands, the body fluids contain less calcium ions than are adequate for health. The bone mineral provides the necessary source of calcium, but the mechanism for dissolving this highly insoluble hydroxyapatite remains an unsolved problem It has been frequently conjectured that some complexing agent of calcium produced locally in bone by a cellular process, might be the solubilizing agent. The ability of citric acid to form a soluble diffusible complex with calcium has been known for a good many years[1-4] as has the presence of some citrate in soft tissues and body fluids[5-7] However it was not until Dickens[8] had demonstrated the presence of citrate in bone in relatively large amounts that attention was focused on determining whether or not this substance actually plays an essential role in mobilizing calcium from the skeleton Its presence in bone had been overlooked previously because nearly all bone analyses were made on ashed specimens.

The present discussion is intended to present a brief résumé of some observations that relate citrate metabolism to that of calcium and bone

Distribution of Citrate. Although the greater part (70 to 90 per cent) of the body's store of citrate is present in the skeleton[8, 9] this amount must represent only a small fraction of the total amount formed and destroyed in the animal organism daily Analyses indicate that the citrate concentration generally ranges from 0 4 to 2 0 per cent of the dry fat free weight of bones The amount of citrate in bone has been shown to vary in different parts of the skeleton and in different parts of the same bone The skeletons of amphibia fish reptiles and mammals all have been shown to contain citric acid (Table 15 1) The spine of teleost fish may contain as much as

Table 15-1

BONE CITRIC ACID VALUES OF SOME BIRDS, FISH AND ANIMALS

Species	No analyses	Citric acid § Gm/100 Gm
Cod spine (*Gadus callarias*)	2	2 01
Herring spine (*Clupea harengus*)	3	5.25
Frog bone	6	0 29
Black-headed gull (*Larus ridibundus*)		
Breast bone	3	2 07 ± 0 44
Humerus	3	0 60 ± 0 06
Egg shells, chicken†	30	0 15
Sea lion‡	1	1 15
Sitatunga	1	0 66
Rabbit	1	0 71
Tapir	1	0 92
Turtle	1	0.31
Baboon	1	0 98
Frog	1	0 26

Thunberg, T Acta physiol. scandinav., 15 39 1948.
† Thunberg T Acta physiol scandinav 17 83 1949 Suppl. 58-60
‡ Author s analyses of femora.
§ Dried fat-free bone.

2 to 5 per cent citrate while frog bones may contain as little as 0 29 per cent citrate [10, 11] The gastroliths of crawfish also contain citrate according to Thunberg [11] Citrate has been demonstrated in a wide variety of calcareous deposits arising in living organisms and has been found in egg shells consisting mainly of calcium carbonate [12-14] Before the relatively high concentration of citrate in the skeleton was known, Kuyper showed that a precipitate of calcium and phosphate formed in the presence of citrate invariably contained the latter substance [15] This observation has been amply verified and extended by other investigators.[16, 49] It must be concluded that the presence of citrate in biologically occurring deposits of calcium is an inevitable consequence of the physical and chemical characteristics of the substances involved The formation of such a highly insoluble complex appears in itself adequately to explain how most of the body s citrate is present in the skeleton This is a stable form of citrate much of which may be relatively inert, since only 5 to 10 per cent can be removed by exhaustive extraction with water according to Dickens [8] and only a part of which appeared to be exchangeable according to the in vitro experiments by Armstrong and Singer [17]

Formation of Citric Acid in the Body The physiologic role of citric acid began to unfold a few years prior to recognition of its occurrence in bone Sherman Mendel and Smith[18] studied the effect of various factors on its excretion in the urine and noted that a definite increase was produced by the

administration of alkali. Orten and Smith[19] demonstrated the marked augmentation of urinary citrate produced by the administration of malic and certain other dicarboxylic acids Krebs, Salvin, and Johnson[20] extended these observations, showing that oxaloacetate had a similar effect to the dicarboxylic acids and that increased excretion of α-ketoglutarate and succinic acid, as well as citrate resulted from injection of these compounds. Based on their own work, that of Szent-Györgyi and others, Krebs and Johnson[21] proposed that the formation and destruction of citric acid take place in living tissue as part of the energy producing mechanism of the body Breusch[22] Green,[23] Ochoa[24] and many others have contributed to isolation and characterization of the enzymes and cofactors concerned in this series of reactions Dixon and Perkins[25] demonstrated that bone, particularly in the metaphyseal zone of young animals, contains the enzymes most immediately concerned in citrate formation and destruction, namely

1 *Citrogenase the condensing enzyme* which catalyzes the reaction of oxaloacetate with pyruvate to form citric acid.

2. *Aconitase* which catalyzes the conversion of citric acid through an intermediate form, aconitic acid

3 *Isocitric dehydrogenase* which does not react with citric acid itself but only with isocitric acid, converting the latter into α-ketoglutarate.

The original observations of Dickens and Perkins indicated that the last mentioned enzyme manifested only weak activity in bone A preliminary report by Van Reen and Losee[26] dealing with experiments in which additional cofactors (Mn and TPN) were included, indicates considerable isocitric dehydrogenase activity in all bone, particularly in the spongy portion. Working in the author's laboratory, Decker has confirmed the high isocitric dehydrogenase activity of the spongy portion of rat bones.[27] It seems evident that bone, freed of marrow contains the enzymes most immediately concerned in the formation and destruction of citric acid, so one need not suppose that the citric acid present in bone is necessarily derived from body fluids, nor has any proof been submitted thus far to indicate that bone citrate is of purely local origin.

Development of a satisfactory method of citrate analysis by Pucher Sherman, and Vickery[28] did much to facilitate studies on the metabolic significance of citric acid Several of the workers referred to earlier also Greenwald,[29] postulated that a small fraction of the diffusible calcium of plasma may exist as a citrate like complex The presence of relatively stable concentrations (2 to 5 mg per 100 cc) of citric acid in the plasma of man and animals has been demonstrated repeatedly[28, 30] However studies on the dissociation of calcium citrate complex by McLean and Hastings,[31] using their frog heart method indicated that slight if any plasma calcium is normally present as a citrate complex Although marked changes in the concentration of circulating citrates have been produced by a variety of experimental procedures the origin, fate and metabolic role of circulating

citrate remain uncertain However there is evidence that the relatively stable plasma concentration of citrate is maintained by a balance between the continuous addition of citrate to and removal of this substance from the circulation

Origin and Fate of Circulating Citric Acid. Comparison of the arteriovenous differences in plasma citrate concentration across various organs or parts of the body indicates that the drop is consistently greatest across the kidney while the citrate content of femoral vein plasma from the dog is usually equal to or higher than in arterial blood The role of the liver in citrate removal from the circulation remains uncertain Hepatectomy of the dog resulted in an increased excretion of citric acid, according to Boothby and Adams [22] Martensson concluded from experimental studies on the rabbit that the liver takes only a small part in metabolizing circulating citric acid. Perfusion of isolated rabbit liver gave little evidence of citrate uptake while similar experiments on the isolated kidney demonstrated a disap-

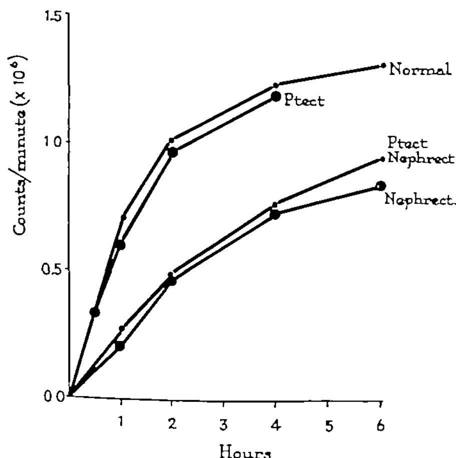


FIG. 15-1 Showing the excretion of C^{14} labeled carbon dioxide following the intraperitoneal injection of 2.5 μc of citric acid 1,5-C^{14} into 100-Gm male rats. Radioactivity of expired air (ordinate) is plotted against time. (Ptect. = parathyroidectomized, nephrect. = nephrectomized) (*Meintzer and Freeman unpublished*)

pearance of citrate from the perfusion fluid.[33, 34] Unpublished experiments by the author failed to demonstrate a consistent increase in the plasma citrate of dogs following complete hepatectomy. Species differences in citrate metabolism suggest that data obtained on animals may not be applicable to man.

Studies in which carboxyl labeled citrate was injected intraperitoneally into rats demonstrated a rapid decline of radioactivity in the plasma accompanied by its appearance in excreted carbon dioxide[35] (Fig. 15-1).

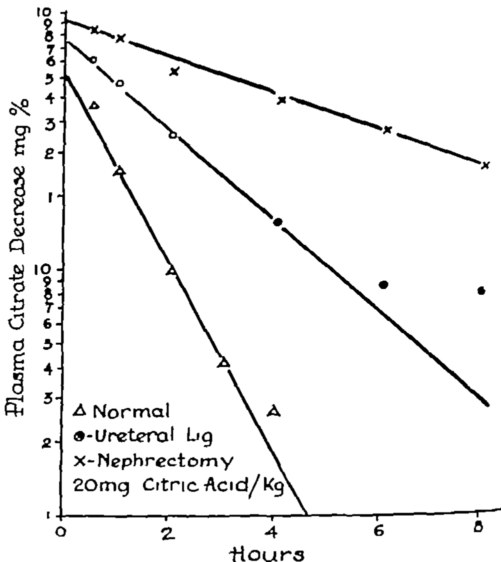


FIG. 15-2. Semilogarithmic plot showing the rate of disappearance of intravenously injected neutralized citric acid (20 mg/kg) from the plasma of male adult dogs. The curves represent average values on 12 normal, 2 ureteral ligated, and 4 nephrectomized animals. The disappearance rate was determined on the operated animals 72 hours after suppression of renal function. (*Kwan and Freeman unpublished.*)

The radioactivity derived from bone 1 hour following citrate injection was similar to what one might have expected from the fluid content of the bone Lussier injected white rats with radioactive citrate and reported 5 per cent of the activity in the skeleton after 1 hour and very little radioactivity in the expired air 4 hours after citrate injection Pretreatment of rats with parathyroid extract increased the citrate uptake by the skeleton although the skeletal activity remained a small proportion of the total activity injected.[26] Herndon and Freeman[27] made in vivo studies of the simultaneous renal blood flow and citrate uptake by the kidney of dogs, which showed that several grams of citrate may be removed daily from the circulation by this organ, an amount far in excess of that excreted in the urine The capacity of the kidney to metabolize circulating citrate is much greater than the actual performance under ordinary conditions Removal of endogenous citrate from the circulation by the kidney was somewhat less in the hypoparathyroid animal presumably because the blood concentration was lower Additional evidence for the importance of the kidney in removing citrate from the circulation was obtained by comparing the rate of citrate removal in normal, ureteral ligated and nephrectomized dogs The disappearance of injected citrate (20 mg/kg) was complete after 3 hours in normal animals and was only slightly prolonged after ureteral ligation

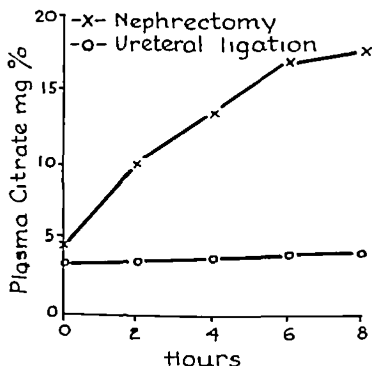


FIG. 15-3 Showing the immediate effect of bilateral nephrectomy or ureteral ligation on the plasma concentration of citric acid in adult male dogs. The curves represent average values on 4 nephrectomized and 2 ureteral ligated animals. (*Kwan and Freeman unpublished*)

while the plasma values in the nephrectomized dogs were still elevated 8 *hours after citrate injection*[19] *(Fig 15 2)*

Factors Affecting the Plasma Citrate Response to Nephrectomy Martensson[24] was the first investigator to demonstrate a rise in the blood concentration of citrate following bilateral nephrectomy The rapid increase in plasma citrate concentration following bilateral nephrectomy in the rat, rabbit, guinea pig, and dog[23] (Fig. 15 3) may be assumed to reflect to some extent the rate of turnover of circulating citric acid occurring in these animals Absence of a similar increase following nephrectomy of the monkey requires further study to determine whether the turnover of circulating citrate is less or the extrarenal metabolism greater in these animals[20]

The effect of various factors on the plasma citrate response to nephrectomy has been studied in rats and to some extent in dogs Without going into experimental details the following statements summarize the findings on fasting albino rats Unless otherwise specified, the rats used were males weighing 300 to 350 Gm

1 The maximum plasma citrate concentration occurs approximately 4 hours after nephrectomy and returns to normal within 24 hours. Evisceration does not abolish this response to nephrectomy

2 The rate of excretion of radioactivity in the expired air of young (150 Gm) fasting nephrectomized rats injected with C^{14}-labeled citrate is clearly *reduced as compared with their intact controls.*[25]

3 Female rats have a significantly greater citrate rise than males, and the plasma value may still be elevated 24 hours after nephrectomy[25]

4 Parathyroidectomy 4 days prior to nephrectomy will significantly reduce or completely abolish the citrate response to nephrectomy in both dogs and rats In the female rat the citrate response to nephrectomy is significantly reduced but is still quite marked after parathyroidectomy The male *parathyroidectomized rat injected with estrogens for 2 weeks prior to nephrectomy shows a response similar to that of the female*[18]

5 Restoring the serum calcium of the parathyroidectomized animal to normal with vitamin D prior to nephrectomy did not restore the citrate response in the dog or rat.

6 Preliminary experiments have shown that vitamin D deficiency in young rats and the administration of toxic doses of vitamin D for 4 days to *adult rats reduced the plasma citrate response to nephrectomy*[23]

7 Parathyroid extract (10 to 40 units per 100 Gm per day) administered for 4 days significantly reduced the citrate response to nephrectomy[23]

From these observations it seems evident that the two factors—vitamin D and parathyroid function—known to influence calcium metabolism as it pertains to the skeleton both affect the citrate response to nephrectomy Lack *of uniformity in their vitamin D content may account for some of the varia*tions in the response to nephrectomy encountered in different batches of

rats procured commercially It is not apparent whether the citrate response to nephrectomy in the parathyroidectomized female rat relates to the skeleton. There is evidence in the literature indicating that certain steroids influence citrate metabolism in placenta[10] and liver[11] and possibly elsewhere[1] Since the evidence thus far obtained shows no effect of parathyroid function on the body s capacity to remove citrate from the circulation it may be assumed that the reduced citrate response to nephrectomy following parathyroidectomy *indicates a reduced rate of citrate ingress into the* circulation The foregoing observations are compatible with the supposition that there is a small labile pool of citrate in the skeleton

Calcium Mobilization by Citrate. Evidence concerning the ability of citrate directly or indirectly to mobilize calcium from the skeleton deserves some consideration. Gomori[13] injected citrate into normal rabbits and demonstrated hypercalcinuria and histologic changes in the skeleton resembling those produced by parathyroid extract He regarded the citrate effect as mediated through the parathyroid glands Chang and Freeman[14] found that citrate infusion will rapidly elevate the plasma and urinary calcium of intact dogs. Attempts to carry out similar experiments on parathyroidectomized animals were unsatisfactory because the animals developed *tetany so readily Exogenous citrate may not be capable of simulating* conditions in which its production takes place within a cell Numerous experiments in the author s laboratory have shown an increase of plasma calcium in association with a hypercitricemia produced by various means in both normal and parathyroidectomized rats Some of these observations may be summarized as follows

1 In both male and female rats, there is in general a parallelism between the plasma citrate and calcium levels following bilateral nephrectomy

2 Parathyroidectomized hypocalcemic female rats show an elevation in both plasma calcium and citrate following nephrectomy

3 Injected fluoroacetate elevated the plasma calcium and citrate of both normal and parathyroidectomized rats

4 Intravenous injection of a toxin* derived from *Aspergillus fumigatus* produced a hypercitricemia and hypercalcemia in female rats 24 hours following its injection The toxin produced a similar degree of hypercitricemia following parathyroidectomy but the increase in plasma calcium was somewhat less in these animals This toxin produced no hypercitricemia or hypercalcemia in the male rat (Table 15 2)

It seems evident that hypercitricemia is associated with calcium mobilization from the skeleton both in intact and parathyroidectomized animals The hypercalcemia associated with hypercitricemia differs from that observed clinically *or that produced by parathyroid extract or large doses of*

* The author is indebted to Dr Evelyn B Tilden, Research Bacteriologist at the Brookfield Zoo, for a generous supply of this toxin.

Table 15-2

CALCIUM AND CITRATE VALUES IN NORMAL AND PARATHYROIDECTOMIZED RATS*

Condition	No rats	Plasma: Citrate mg/100 cc average S.D	Plasma: Calcium, mg/100cc average S D
Female			
Normal	14	6.0 ± 2.7	10.16 ± 0.32
Pth†	14	5.7 ± 1.4	0.04 ± 1.17
Neph‡	14	38.45 ± 3.6	13.65 ± 0.56
Pth, Neph	7	20.0 ± 5.1	9.9 ± 1.5
Toxin§ normal	11	75.0 ± 17.4	10.8 ± 1.71
Toxin Pth	11	76.0 ± 17.4	11.8 ± 0.0
Male			
Toxin	7	5.3 ± 0.02	10.4 ± 0.96

* Body weight 300 to 350 Gm.
† Pth = parathyroidectomized 9 to 14 days before bleeding.
‡ Neph = nephrectomized 4 hours before bleeding
§ Toxin of *Aspergillus fumigatus* injected intravenously 24 hours before bleeding.

vitamin D in that the citrate levels are relatively much higher and tetany may be manifest. However there is some experimental evidence to indicate that altered citrate metabolism may elevate the plasma calcium concentration without the occurrence of high plasma concentrations of citric acid. According to Buffa and Peters,[43] fluoroacetate forms fluorocitrate in the body which in turn blocks citrate oxidation so that citrate accumulates in the tissues Large doses of fluoroacetate (1 to 5 mg/kg) will clearly increase the citrate content of the blood as well as of the tissues in the rat. The dog is exceedingly sensitive to fluoroacetate and fatal convulsions may be produced by even very small amounts of fluoroacetate (25 to 50 γ/kg) In the author's laboratory two dogs given 25 γ/kg of sodium fluoroacetate that did not have convulsions developed a hypercalcemia, which was unaccompanied by any marked change in plasma concentration of citrate (Table 15 3) Perhaps the increased citrate content of some tissue reduced its calcium ion content and thereby stimulated parathyroid function or the effect could have been a direct one on the cells of bone trabeculae. Efforts to potentiate the hypercalcemic effect of parathyroid hormone by fluoroacetate thus far have been unsuccessful Studies on parathyroidectomized dogs are handicapped by their extreme sensitivity to fluoroacetate

Relation between Plasma Concentration of Calcium and Citric Acid. Various authors have called attention to an apparent relation between the plasma concentration of calcium and citric acid. Hypocalcemia produced by parathyroidectomy is associated with a reduced concentration of circulating citric acid [44] and the same is true of experimentally induced vitamin

Table 13

EFFECT OF SODIUM FLUOROACETATE ([illegible] γ PER KG I.V.) ON PLASMA CALCIUM, PHOSPHORUS AND CITRIC ACID OF FEMALE DOGS

	Time, hr	Mg per 100 cc		
		Citric acid	*Calcium*	*Phosphorus*
No. 1	0	6.7	11.0	4.8
	0.5	6.0	11.5	4.6
	1	7.4	11.2	4.8
	2	7.4	12.1	5.7
	4	7.4	13.1	5.7
	8	0	13.8	14.5
	24	6.1	10.8	5.7
No. 2	0	- -	10.8	4.5
	0.5	8.0	11.8	4.5
	1	- -	12.3	4.5
	2	7.7	12.2	4.5
	4	10.3	13.2	4.6
	8	9.8	13.0	0.0
	24	7.7	10.0	4.8

D deficiency.[47] Harrison[48] reported that vitamin D deficiency in children and rats is accompanied by low plasma citrate values irrespective of the levels of serum calcium. Previously published[39] data from this laboratory showed that a prolonged hypercalcemia in dogs produced by giving parathyroid extract, is associated with slightly less hypercitricemia than when a similar hypercalcemia is produced by vitamin D administration. Other data indicate that a single dose of parathyroid extract sufficient to produce marked hypercalcemia (18 to 22 mg per 100 cc) in 16 to 24 hours does not always raise the plasma citrate level significantly in dogs. Injection of parathyroid extract into hypoparathyroid dogs may actually produce a decrease in the plasma citrate concentration at the same time that the serum calcium level is rising. As pointed out by Neuman[49] and observed in our own studies, some of these variations depend upon the time of blood sampling following administration of parathyroid extract. This topic will be referred to later. It seems reasonable to conclude that factors other than chemical affinity or mutual stabilization of calcium and citrate must enter into regulating the citrate concentration of the circulation.

Factors Affecting the Citric Acid Content of Bone. A few experimental studies have been carried out, mostly on the rat, to demonstrate whether or not the citrate content of bone can be altered. Class and Smith[13] found 5 to 7 mg of citrate per gram of wet bone or 70 to 80 mg per skeleton in a 230- to 240-Gm rat. A high urinary excretion of citrate provoked by alkali or malate administration was without effect on bone citrate. Thompson[14] analyzed the shaft and ends of long bones separately and found that the

Table 15-4

CALCIUM/CITRIC ACID RATIO IN RAT BONE

Group	Description	No. rats	Calcium/citric acid ratio	Groups compared	Probability
			meq		
Female					
I	Control	15	135.22 ± 16.8	I and II	< 01
II	Toxin†	10	108.00 ± 10.34	III and IV	> 7 < .8
III	Pth‡	14	100.86 ± 14.01	I and III	< .01
IV	Pth, toxin	20	111.55 ± 16.71	II and IV	> 4 < .5
Male					
I	Control	14	1?4.45 ± 10 12	I and II	>.3 < 4
II	Neph§	15	12? 01 ± 10.27		

Rats weight 200 to 220 Gm. The entire right tibia and femur of each animal was dissolved in acid and used for calcium and citrate analysis.

† *Toxin of Aspergillus fumigatus* injected intravenously 24 hours before sacrificed.

‡ Pth = parathyroidectomized 2 weeks before sacrificed.

§ Neph = nephrectomized 24 hours before sacrificed. Significant reduction of per cent ash in nephrectomized group

calcium/citrate ratio was lower at the ends of the bones. In growing rats the absolute concentration of citrate was similar in the two regions The calcium/citrate ratio in the metaphysis *remained constant throughout life*, while there was a threefold increase in the calcium/citrate ratio of the shaft in old animals Several investigators have reported that vitamin D deficiency reduces the citrate content of bone[8, 47, 50, 51] Steenbock and Bellin[47] showed that vitamin D increases the citrate content of all tissues in the vitamin D deficient rat. Dickens[8] reported a 27 per cent increase in the bone citrate of a puppy given parathyroid extract for several weeks. Perkins and Dixon[52] reported no difference in the bone citrate levels of normal animals and those parathyroidectomized 4 weeks previously Current studies in the author's laboratory have shown a statistically significant decrease in the calcium/citrate ratio of the femur and tibia of female rats 2 weeks following parathyroidectomy This change is due to an increase in the citrate content of the bone without a corresponding increase in its calcium content. A similar change in the bone calcium/citrate ratio was produced in female rats by injection of toxin† derived from *Aspergillus fumigatus* The toxin plus parathyroidectomy produced no further effect than that produced by either one alone Bilateral nephrectomy of 200-Gm rats failed to alter the calcium citrate ratio of long bones after 24 hours although there was a significant decrease in the per cent ash contained in the bone[53] (Table 15-4) Experiments concerning the effect of fluoroacetate upon the citrate

† In an in vitro experiment, this toxin suppressed citrate oxidation and oxygen consumption by rat kidney mitochondria.

content of bone have led to conflicting results.[16,31,35] In these and other studies significant changes in metabolically active citrate may be obscured by the presence in bone of relatively large amounts of storage citrate.

Parathyroid Function, Plasma Citrate, and Mobilization of Calcium from the Skeleton. In vitro studies have shown that citrate tends to have a solubilizing effect upon calcium phosphate and upon powdered bone.[16,40] Neuman and collaborators[49] have reported on in vivo studies intended to demonstrate any association that may exist between calcium mobilization and the liberation of citrate from the skeleton. Their results show that, following administration of parathyroid extract intravenously, blood derived from the metaphysis of a bone may have a higher citrate content than previously and that a rise in the external jugular vein concentration of citrate precedes the elevation of serum calcium produced in dogs by parathyroid extract. Similar experiments in the author's laboratory intended to relate the citrate content of arterial and bone blood of parathyroidectomized dogs have in some instances yielded similar results to those mentioned above while in other instances parathyroid extract appeared to produce a definite decline in the plasma concentration of citrate even though the calcium level was rising. Hormone dosage, route of administration, and the timing of blood samples may be factors in producing the differences in results. For example in three experiments arterial as well as metaphyseal, blood samples from the femur and humerus clearly showed a decrease in citrate concentration 22 hours after injection of parathyroid extract, while in another experiment blood drawn 2 and 4 hours after hormone administration showed a rise in the plasma citrate level, particularly in the metaphyseal samples (Fig 15-4). The rate of blood flow from a hole drilled into the spongiosa of a long bone varies greatly and may be markedly influenced by factors affecting the systemic circulation. Based on strontium uptake by bone Firschein and collaborators[49] have estimated that a fourth or fifth of the blood so collected contacts bone trabeculae. The apparently paradoxical effects of parathyroid extract on the level of blood citrate may be presumed to relate to calcium mobilization, but give no clue as to what role citric acid may have in this process.

Enzyme Activity in Relation to Vitamin D and Parathyroid Function. There is only meager information concerning the effect of vitamin D or parathyroid extract upon enzyme systems that may be concerned in the formation or destruction of citric acid. Perkins and Dixon[52] reported a decrease in the bone citrogenase of parathyroidectomized animals. The activity of this enzyme was not restored to the normal range by hormone administration. Laskin and Engel[54] reported a reduced oxygen consumption by metaphyseal bone slices following the administration of parathyroid extract to rabbits. This effect was attributed to diminished succinic dehydrogenase activity. Efforts by Fried in the author's laboratory to demonstrate any effect of parathyroid extract on citrate oxidation by preparations

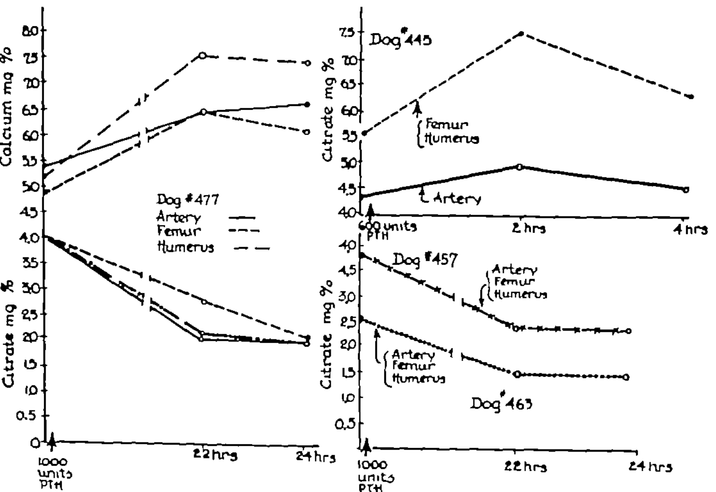


Fig 15-4 Showing the effect of parathyroid extract on the plasma citrate concentration in samples of blood obtained almost simultaneously from an artery and from holes drilled in the metaphyseal region of long bones in thyroparathyroidectomized fasting adult mongrel dogs. Plasma calcium values obtained on the same samples are shown for dog No 477

of kidney mitochondria from normal or parathyroidectomized rats have been entirely negative [17] DeLuca Gran and Steenbock [8] showed that the addition of vitamin D to kidney mitochondria from rachitic rats decreased the oxidation of citrate and isocitrate to α-ketoglutarate Bruchmann [9] has recently reported a marked inhibitory effect of vitamin D_2 upon the activity of aconitase obtained from *Aspergillus niger*

Citrate in Relation to Calcium of Soft Tissues. DeLuca and collaborators[8] reported that the addition of calcium to kidney homogenate reduced citrate oxidation On the contrary Perkins[60] states that the addition of calcium, even in amounts that are equivalent to the citrate added was without effect on citrate oxidation by minced kidney Activation of myokinase by calcium ion has been reported [61] Magnesium is a cofactor for several of the Krebs cycle enzymes and its effect may be modified by the concentration of calcium ions The body must possess a highly effective mechanism for maintaining the very low concentration of calcium found in most cells. Little is known concerning the possible role of citrate in the transport of calcium across cell boundaries or in stabilizing calcium ion concentration in the tissues. It seems plausible to suppose that the extreme sensitivity of nervous and muscular structures to calcium ion concentration must somehow regulate parathyroid function hence calcium mobilization from the skeleton

Summary and Conclusions

Citric acid is continually entering and leaving the circulation of certain animals and presumably that of man It is probable that part of the circulating citrate originates in the skeleton and is metabolized by the kidney Vitamin D and parathyroid extract can influence the citrate content of plasma and bone, but it is not evident how this effect relates to calcium metabolism There is evidence that male and female animals have qualitative and quantitative differences in citrate metabolism In vivo and in vitro findings indicate that steroid hormones may influence the formation and destruction of citric acid. The citrate content or the calcium/citrate ratio of bone may vary with the species, age sex and site from which the bone sample is taken. The calcium/citrate ratio of bone has been altered experimentally In general, this ratio is lowest in those parts of the skeleton which one would expect to be most active metabolically Physical and chemical characteristics of calcium, phosphate and citric acid account for the high citrate content of the skeleton, but fail adequately to explain variations in the calcium/citrate ratio occurring naturally or produced experimentally It remains to be established whether or not citric acid plays an essential role in the mobilization of calcium from the skeleton or in regulating calcium ion content or transport in the soft tissues of the body

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16

Phosphorus Metabolism*

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For many years phosphorus was cast in the role of a stepchild of calcium. A few years ago it was enough to say of both elements that calcium and phosphorus are necessary in quite large amounts to provide calcium phosphate—the important salt of which the bones are formed

The new era in phosphorus metabolism was in the decade beginning in 1926 and was signalized by demonstration of the importance of phosphorus compounds for energy transfer It was during this period that phosphocreatine and adenosine triphosphate were first isolated and identified and that the high-energy phosphate bonds of these compounds were observed by measuring the heat of dephosphorylation.

The tremendous increase in interest in phosphorus in the past thirty years is familiar to every chemist and biological scientist. It would be manifestly impossible in the time allotted to me even to list the reactions in which phosphorus participates in the living organism and the vital functions with which it is concerned. This is a symposium on bone One result of the work of recent years has been to relegate the participation of phosphorus in calcification to a relatively insignificant place Our task for today is to attempt to rescue it from its obscurity and to place it in what we hope will be a proper perspective This will require attention to some generalizations

In the first place it appears that there is no homeostatic mechanism directed primarily toward the control of levels of phosphorus compounds in the blood or toward the conservation of phosphorus by the organism as a whole This latter fact has not been sufficiently emphasized. In the face of myriads of competing demands for phosphorus for utilization in reactions essential to life and health, the organism wastes phosphorus in a seemingly

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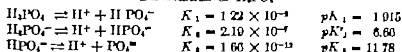
uncontrolled way its only protection lying in the fact that for every molecule wasted another turns up in the diet Whereas calcium deficiency is the rule, even in diets excessive in caloric intake the opposite is true for phosphorus Diets truly deficient in phosphorus are rare indeed, and when they exist they are invariably characterized by other deficiencies such as those of vitamins and/or proteins so that one can observe the specific effects of phosphorus deficiency only under the most rigorous experimental conditions.

Some years ago I had the privilege of studying with Dr Smith Freeman the effects of what amounted to virtually complete phosphorus deficiency in puppies, both in the presence and absence of vitamin D[1] Regardless of whether vitamin D was given florid rickets developed associated with a striking decrease in the inorganic phosphate levels in the serum and whole blood of the animals Skeletal calcification ceased entirely On the other hand, the acid-soluble organic phosphorus content of the blood was never significantly altered.

This study revealed that the organism *does* possess a mechanism capable of protecting the needs for phosphorus in reactions essential to life In the puppies, life was maintained by shutting off the largest demand for phosphorus—that needed for calcification. What remained, even under these extreme conditions was adequate to fill the supply lines of acid-soluble organic phosphorus compounds and this situation was made possible only by denying any phosphorus to the skeleton. To this extent the phosphorus of the organism is conserved to the degree required by the basic needs of the soft tissues and this constitutes a contradiction in terms at least, to what we said a moment ago It also demonstrates that calcification, itself not immediately essential to life enjoys only a low priority with respect to such phosphorus as is available to the organism

Transport of Phosphate in the Organism. Let us pause for a moment to consider some of the properties of the compounds of phosphorus found in the animal organism. First, nearly all the phosphorus in the body is in the form of the ions of orthophosphoric acid H_3PO_4 A smaller amount exists as derivatives of pyrophosphoric acid, $H_4P_2O_7$ itself a condensation of two molecules of orthophosphoric acid. We shall have more to say about this fraction later

Of the 6 mg of phosphorus per 100 cc of plasma, all but approximately 0.5 mg is inorganic, as the ions of orthophosphoric acid chiefly as $HPO_4^{=}$ The tabulation which follows illustrates the dissociation constants of phosphoric acid and shows that the second dissociation constant, or pK_2 is 6 66 indicating that at pH 6 66 one half of the phosphate is in the form of $HPO_4^{=}$ From these constants it is calculated that at pH 7 4 approximately 85 per cent of the total inorganic phosphate of the plasma is present as the divalent ion $HPO_4^{=}$ while 15 per cent is monovalent $H_2PO_4^{-}$ and only 0 0035 per cent is trivalent $PO_4^{\equiv}$



Dissociation of H_3PO_4

$H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^-$	$K_1 = 1.22 \times 10^{-2}$	$pK_1 = 1.915$
$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{=}$	$K_2 = 2.19 \times 10^{-7}$	$pK_2 = 6.60$
$HPO_4^{=} \rightleftharpoons H^+ + PO_4^{\equiv}$	$K_3 = 1.66 \times 10^{-12}$	$pK_3 = 11.78$

Although there is only a small amount of organic phosphate compounds in the plasma, the red blood cells are rich in organic acid-soluble compounds of phosphate the evidence is to the effect that these are not simply fixed substances in transit. They are labile and are engaged in the transformations typical of similar compounds elsewhere and specifically in anaerobic glycolysis within the red cells themselves

An important development of the past few years has been clarification of the mode of transport of phosphate in the blood of laying birds, fish, amphibians, and reptiles In birds during the egg-laying cycle a phosphoprotein called serum vitellin appears in the plasma, and this is associated with a pronounced hypercalcemia levels of 20 to 50 mg of calcium per 100 cc of plasma being common, and higher levels being attained when estrogens are administered, even to roosters Urist and his collaborators[2] have shown clearly that the function of the phosphoprotein is to transport phosphate for storage in the egg yolk. The excess of calcium in the plasma is in combination with the phosphoprotein the level of ultrafilterable calcium is not increased That this mechanism serves primarily to transport phosphate rather than calcium, is shown by the fact that the conditions in the plasma are the same in fish, amphibians and reptiles as in birds and that, of these species, only birds produce eggs with calcified shells.

Synthesis of Organic Phosphate Compounds. A major factor in the metabolism of phosphorus is that the organism is able to synthesize all its many complex organic compounds of phosphorus from inorganic phosphate This is not to say that it can synthesize all the organic substances with which phosphate is united. The human organism, for example, cannot synthesize thiamine But given thiamine the organism can transfer it into the active form *thiamine pyrophosphate Moreover phosphate ingested* in organic combination is for the most part split off and absorbed in the same manner as if ingested as inorganic phosphate The biosynthesis of the large number of phosphorus-containing catalysts and intermediates requires additional enzyme systems many of which themselves contain phosphorus Few such biosyntheses have been traced back to their earliest stages

I have already said that no homeostatic mechanism directed primarily toward the control of levels of phosphate compounds in the blood is known. Various efforts have been made to link this function with the parathyroid glands but with indifferent success The body calls on its stores of calcium through the parathyroid glands, by virtue of a feed-back mechanism which controls parathyroid activity [3] Whenever parathyroid activity is increased,

bone mineral is mobilized liberating both calcium and phosphate into the circulation But while this results in an increase in the concentration of calcium ions in the plasma it usually is accompanied by a decrease in the phosphate level rather than by an increase This depends upon a secondary and independent effect of the parathyroid hormone upon reabsorption of phosphate by the renal tubules, its net effect is not to increase the amount of phosphate available for metabolic activities but rather to increase its wastage

Enzymatic Factors in Calcification The role of enzymes in calcification remains to be clarified One thing however seems to be certain Wherever enzymes may be implicated in the calcification process phosphate containing organic compounds will also be found

Robison, in 1923 [4] discovered an alkaline phosphatase in hypertrophic cartilage and developing bone and noted that its site and concentration were correlated with the onset and extent of calcification in these tissues He suggested that this enzyme acts upon some phosphoric acid ester substrate to produce a state of local supersaturation of the interstitial fluids of calcifying tissues, with consequent precipitation of the bone salt

It is hardly necessary to review the early history of this subject, or to point out that Robison himself abandoned his initial simple hypothesis in favor of a more complicated version in which he introduced the concept of a second mechanism never adequately clarified, but believed by Robison to be enzymatic in character What is important to note now is that Robison s discovery preceded the isolation of phosphocreatine and of adenosine triphosphate and the demonstration of high-energy phosphate bonds in these and other compounds of phosphorus Robison s work, and that of many who have followed his lead, was based on concepts of the metabolism of phosphorus since outmoded. It was however suggested as early as 1932[5] that the appearance of glycogen in hypertrophic cartilage and its disappearance prior to or during calcification were in some way related to the calcification process, perhaps as a mechanism for endogenous production in cartilage of phosphoric acid esters to furnish a substrate for phosphatase

This idea was later improved upon especially by Gutman and Yu[6] and Marks and Shorr [7] who demonstrated that the glycolytic cycle beginning with phosphorylative glycogenolysis in the hypertrophic cartilage cells plays an important, and in some respects a decisive role in calcification of cartilage matrix Every step in the glycolytic cycle until pyruvate is formed, includes reactions in which phosphate is involved in one way or another this forms a modern link with the earlier views of Robison Unfortunately however the idea that the glycolytic cycle contributes to the accumulation of phosphate to be later made available for calcification has not been supported if only for the reason that with pyruvate the cycle runs into a dead end, in so far as production of esters available for alkaline phos

phatase is concerned Gutman and Yu improved upon this idea by suggesting that, instead of functioning as a hydrolytic agent to furnish inorganic orthophosphate, phosphatase may serve in the enzymatic transfer of a phosphate group from a metabolic ester to an acceptor in the matrix.[8] This, then, proposes a possible role of phosphatase in the nucleation of bone *mineral, instead of requiring it to produce phosphate ions for the further* progress of calcification

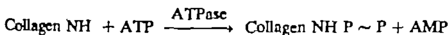
An even more recent development has centered around the possible role of adenosine triphosphate (ATP) in calcification. *Gutman and Yu reported* that they were unable to induce calcification in rachitic cartilage in vitro when phosphate was supplied in the medium only in the form of ATP[4] They stated, however in view of certain technical difficulties, that "We are, therefore not in a position either to affirm or to deny that ATP plays a significant role in endochondral calcification" At about the same time Cartier and Picard reported that, while inorganic phosphate or hexose phosphates led to very poor mineralization of embryonic cartilage in vitro very heavy mineralization was induced if phosphate was supplied as ATP[8] This finding was confirmed, as late as 1958 by Perkins and Walker in the cartilage of rachitic rats[9] Thus ATP which has been called the *unit of currency* in metabolic energy transformations, now seems to be related to the calcification process although the nature of this relationship is by no *means clear*

In the wake of ATP however another compound—pyrophosphate—has entered the puzzle of calcification Until recently pyrophosphate had been found in mammalian tissues in only very small amounts, in such organic compounds as thiamine pyrophosphate Cartier and Picard, however found that when phosphate was supplied to embryonic sheep cartilage in the form of ATP approximately 80 per cent of the phosphate deposited in the cartilage was pyrophosphate[10] The corresponding figure for rachitic rat cartilage was found by Perkins and Walker to be only about 5 per cent they attributed the difference to a difference in the inorganic pyrophosphatase activity of the two tissues[9] Perkins and Walker also reported, for the first time the finding of pyrophosphate in normal bone and found that the deposit formed in vitro on incubation of rachitic cartilage with a calcifying *medium does not contain pyrophosphate unless* ATP is added to the substrate, when the proportion of pyrophosphate attains the same order as that in normal bone.

The metabolism of polyphosphates has been studied in various lower forms of which the most interesting to us is its accumulation in the cells of bakers yeast, previously deprived of phosphate for 24 hours and then put into a phosphate-containing medium[11] Under these conditions and when an energy source is supplied and potassium ions are present, very large amounts of orthophosphate are absorbed by the cells and condensed to metaphosphate This results in storage of both phosphorus and of energy

We are a long way from linking the storage of energy and phosphate in yeast cells, as metaphosphate to the deposition of pyrophosphate in bone and in embryonic and rachitic cartilage There seems to be abundant reason, however to speculate on the possibility that either calcification itself or some of the events leading up to it may depend upon enzymatic reactions of which we are not yet aware

We have already referred to the proposal of Gutman and Yu that alkaline phosphatase may serve in the enzymatic transfer of a phosphate group from a metabolic ester to an acceptor in the matrix Picard has progressed a step further by proposing the hypothesis that mineralization of cartilage is an enzymatic cellular reaction [12] In support of this he points out that cartilage is the seat of active metabolic reactions, and he believes that these reactions orient its mineralization Cartier has demonstrated the presence of an enzyme in cartilage which he calls ATPase [13] His view is that, under the influence of this enzyme ATP transfers pyrophosphate to some component of the organic matrix, presumably by a reaction with the free amino groups of collagen as follows



$$\text{Collagen NH} + \text{ATP} \xrightarrow{\text{ATPase}} \text{Collagen NH P} \sim \text{P} + \text{AMP}$$

This fixation of pyrophosphate according to Cartier leads to nucleation followed by combination with calcium and by formation of the bone mineral. Since the transfer of a pyrophosphate radical is not a frequent occurrence Zambotti has suggested that this might be a reaction characteristic of preosseous tissue [14] This view is supported by the observation of Solomons and Irving that the availability of the amino groups of lysine and of hydroxylysine increased from 6 per cent in untreated human dentin to more than 90 per cent in fully demineralized dentin leading them to suggest that these amino groups may play a part in the combination of mineral material with the protein matrix.[15]

Sobel and Burger have reported that preliminary treatment of rachitic bone slices with ATP and Ca^{++} led to calcification of both the cartilage matrix and the osteoid tissue at low $Ca \times P$ products [16] Contrary to the reports of Cartier and Picard and of Perkins and Walker they found that ATP when placed in the calcifying medium exerted an inhibitory effect upon calcification They suggest the possibility that preliminary treatment with ATP enhances the activity of the calcifying mechanism by providing energy

No one has yet shown clearly that energy supplied locally is essential to the deposition of bone mineral If this could be demonstrated, the presence of ATP as the *unit of currency* in metabolic energy transformations might assume new significance This however will still require accounting for the fact that reactions in which ATP participates are mainly intracellular while calcification is an extracellular process

Perhaps we can go no further at this time than to suggest that there is

now reason to believe that phosphate heretofore regarded as the passive member of the calcium phosphorus team in calcification may be found to provide such activity or energy as may be necessary for this process, or more particularly for its initiation or seeding In this case the passive or secondary role may have to be assigned to calcium

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DISCUSSION

Vitamin D Parathyroids, Citric Acid, Calcium, and Phosphorus

Chairman John Eager Howard M.D

CHAIRMAN HOWARD Franklin, while your subject is still fresh in our minds why don't you start off with the discussion?

DR. MCLEAN The only question I have here is from Dr Sobel about the paper of Perkins and Walker—when it was published and in what journal

The paper came out in May 1958 [9] The authors did chemical determinations of pyrophosphate They studied calcification of rachitic rat cartilage in vitro and found that when orthophosphate was supplied they were unable to demonstrate pyrophosphate in the calcified cartilage When they used AP in the medium, pyrophosphate was deposited in the cartilage They also demonstrated pyrophosphate in normal rat bone

CHAIRMAN HOWARD Dr Freeman do you have a question?

DR. FREEMAN I have a question by Dr Austin as to the best laboratory method for the determination of plasma citric acid I don t know whether one could say which is the best method Most of the methods depend upon converting citric acid to pentabromacetone The method of Pucher Sherman and Vickery[2] as modified by Hunter and Leloir[3] is satisfactory We use the Ettinger et al [4] modification of this method for plasma because it is more sensitive The method of Natelson Pincus and Lugovoy[5] is also satisfactory and both are considerably more sensitive than the original method. The method as described by Taylor[6] may have some advantages as applied to the larger amounts of citrate encountered in bone

The important thing is that you have to run everything in duplicate that you have to run blanks, that you have to be extremely careful about your solvents and about your timing after you make your extraction with the solvents, because the color does fade I think this is a common experience regardless of which method you use

The next question is Do you regard citrate as an adventitious component of bone salt?"

As I tried to point out probably the greater portion of the citrate in bone is relatively inert, and I suppose one might say that to this extent this might be regarded as adventitious citrate However I do think that there is a small portion of the citrate in bone which is labile and is reactive and which may be related to the immediate mobilization of a relatively small fraction of the calcium in bone I think that the studies that are made with fluoroacetate or other agents in which one attempts to modify the citrate in bone are handicapped by virtue of the fact that the small metabolically active citrate that you are really interested in is diluted down by this relatively large pool of inert citrate

But I do not think that all the citrate in bone can be regarded as adventitious and inert. Perhaps a good deal of it is fortuitous in so far as it reflects any role that citrate may play in the mobilization of calcium from the skeleton.

DR. MCLEAN This word came from Armstrong and what he meant by "adventitious was that it was fortuitous that it just happened to be there because it was in the blood and was picked up by the bone

I think you partly answered that question in your paper by saying you didn't know

DR. HARRISON Obviously no one knows the answer I think part of the citrate in bone just happens to be there because it complexes with calcium which is in the bone crystal and that the concentrations of citrate in bone salt can be influenced to some extent by changing the concentrations of citrate in extracellular fluid There is a difference of opinion on that point, and further studies will have to be done to settle that

DR. FREEMAN I would like to make one more comment Dr Thompson

who is a student of Dr Hastings and who has done quite a little work on the distribution of citric acid in bone has studied proportions between the shaft and the ends of the long bones, and in her work she showed that the ratio of calcium to citrate in the shaft goes up with age—in other words, there is relatively less citrate—whereas, in the ends of the long bones, where the trabeculations are occurring and being formed and reabsorbed most actively the ratio of calcium to citrate is lower and tends to remain more constant throughout life. I do not think, therefore that one can dismiss all the citrate on the supposition that it is merely a storage item

DR HARRISON Dr Bachra asks this question He says "(1) Cortisol prevents the effect of vitamin D in raising serum citrate (2) Cortisol stimulates the movement of citrate to extracellular fluid Are 1 and 2 compatible?"

The answer is no What I said was that cortisol inhibits the movement of citrate to extracellular fluid and then 1 and 2 are very compatible The whole point is that the effect of cortisol which we were able to study in a variety of situations seemed to be inhibition of the flux of citrate from intracellular to extracellular fluid, and in that way it perhaps reduces or prevents the effect of vitamin D on serum citrate

The second question from Dr Winters "In terms of your hypothesis about the effect of acidosis upon vitamin D action on renal phosphorus transport, (1) do you have measurements of TMP in acidotic humans and (2) comment upon the previous data available in dogs bearing upon this point

We do have measurements of TMP (tubular transport of phosphorus) in infants or children with renal tubular acidosis The tubular maximum is markedly reduced during the period of acidosis

We also have measurements in dogs and so does Pitts Pitts and we are somewhat at variance in our experiments We worked with puppies. Being a pediatrician, I am very much biased in favor of the young growing animal *Pitts being a physiologist, is very much biased in favor of the large* adult dog which he can study more easily and maybe there are differences in the reactivity of these animals

We found a reduction of TMP by induced acidosis. Pitts at first said he could not find this in dogs, and then he reported that he found an effect of acidosis in dogs on tubular reabsorption of phosphate but it was somewhat different from the effect that we described It was not an effect upon the maximum tubular transport but on the curve In other words in theoretical terms if this is the amount of phosphorus filtered, if the tubular transport of phosphorus were fitted into a theoretical system, it would follow along the amount filtered until a maximum was reached and then it would flatten off This is the concept of tubular maximum Pitts reported that in the acidotic dog the curve was altered so that the maximum was not reached

promptly, and in this area there was a marked depression of tubular reabsorption of phosphorus

I don't think it is terribly important whether you talk about the tubular maximum or depression of the reabsorption curve The important point that occurs in the living animal is depression of tubular reabsorption of phosphate so that phosphate is wasted in the urine under conditions of acidosis and hypophosphatemia results. Clinical experience indicates that this is the case.

Finally there are a couple of questions that I think have been submitted here, and no one quite knows who should be responsible I shall try to answer the first one "Does ionized calcium have a peripheral vasoconstrictive action?"

Probably Dr Howard should answer that We know that, in hypercalcemic states, hypertension does occur An acute hypertensive encephalopathy is one of the manifestations of calcium poisoning produced either by vitamin D intoxication or by hyperparathyroidism I think Dr Howard ought to answer that question, however

CHAIRMAN HOWARD I would say that it has a vasodilator effect. When you put calcium into the serum, into the vein you get a marked peripheral vasodilatation

DR. HARRISON Someone asks "What influence do cholesterol and chloride have upon deposition of calcium in tissue?

I am afraid I don t know

DR. COPP I think that the last question was a question for a pathologist I imagine that it refers to the deposition of calcium in degenerative areas or cholesterol plaques Well I am a physiologist, so I will duck this one

There is a note of skepticism here raised by Dr Smith Freeman. He says, "A dog usually develops tetany 3 or 4 days after the parathyroids are removed." I presume that this is a Chicago dog "Your data show that the serum calcium drops to tetanic levels in 10 hours How do you account for the lag in the development of tetany?"

First, we very rarely see tetany in our dogs I assume that this is due to the good living and the relaxed atmosphere in British Columbia When it does occur it occurs in a dog which in the postoperative state and in discontent with the surgeon, refuses to eat. We may have to give calcium then, or parathyroid extracts for a day or two until it recovers its good disposition and its appetite After that we do not see tetany in animals even though the blood calcium level is running around 4.5 to 5 mg per 100 cc

The second is a question from Dr Talmage "The latent effect of crude extract—is this not necessarily the true hormone?"

This is a note of skepticism about Eli Lilly company s preparation I think what Dr Talmage is referring to is the fact that in our animals the calcium in the blood kept on going up after we stopped perfusing the stuff

whereas if you take the parathyroids out of a pig or a rat the blood calcium level drops like a stone and within an hour or two it is down to the parathyroidectomized level.

I think that this is a species difference In dogs we find that the blood level following parathyroidectomy doesn t begin to drop for about 2 to 4 hours after the glands are removed at least in adult dogs We find the same period before the blood calcium drops after we stop the infusion of a maintenance dose of 0 1 unit.

So I think that there is parathyroid hormone in the Eli Lilly extract.

I have here a question from Marshall Urist, also about this peculiar substance which is marketed and used as a parathyroid hormone "Is there a parathyroid hormone for the kidney in addition to the one for bone?"

The extract of *Eli Lilly & Co* has an effect on both kidney and the skeleton on both the blood calcium level and the phosphate level in the urine I think that, until we have a chance to use Dr Rasmussen's super pure preparation it will be difficult to say whether there are actually two distinct hormones I understand (and I would like someone from the floor to comment) that there is a phosphaturic effect in Rasmussen s preparation *Is that true?*

(A member of the audience indicated affirmatively)

DR. COPP So it is possible we can still be Unitarians

DR. DORSCH "It was stated yesterday that dietary calcium will not affect the blood calcium level Do you feel that the parathyroid activity is rapid or sensitive enough to account for this?"

Yes

"Are there any opinions with regard to the possible effect of hypophysectomy on the treatment of parathyroidectomized animals with PTE?" Well this is a wonderful experiment and we will just have to set this thing up sometime next year unless Dr Resnide would like to do it first.

Finally there is a question from Dr Comar "You mentioned a membrane Please elaborate "

There are three possible membranes a biological membrane which has cellular activity a capillary membrane and a membrane around each crystal of bone salt because of the accumulated garbage ions (calcium, phosphate citrate carbonate) accumulated on the surface This might be called a sort of garbage membrane

I would like to pass this on to our chairman who is an expert on membranes.

CHAIRMAN HOWARD In the few minutes remaining, I would like to make some comments The moderator is chosen because he has been in the field a long while and should have something new to say but since this is not the case he is given the opportunity to vent his soul in philosophical remarks known as summing up

Many years ago Dr Welch is said to have remarked that every 10 years

experimental approach should stop and a 10-year period should be allowed to digest and assimilate accumulated factual knowledge I suspect that the bone field is ready for that, although things have been so exciting that perhaps we had better wait another year before this period of digestion

Over the past 10 years enormous strides have been made by our physicist and chemist friends, but most clinicians and medical physiologists appear to approach the skeleton through the old concepts Ultimately basic science will provide the final answers as to how things take place but there are it seems to me two roles that the clinician can play in research one, to suggest reasons as to why the "how" is initiated and second to test his more scientific confreres findings to see if these are applicable in the intact human organism for if they are not, they are useless so far as medical science is concerned

It is high time that the clinicians provide some fresh approaches to the study of bone physiology I don t mean in the form of techniques but in intellectual approaches The title of this symposium on Bone as a Tissue provides one such approach for tissue is a mass of cells which perform specific functions To the bone cells we allocate an enormous number of jobs, as Dr Gersh has said to grow to nourish itself to provide a collagen structure with mineral deposition, to destroy itself in toto all these things under a variety of influences of course

But the concept more and more emerges that the cells themselves accomplish all these things Environmental hormone and other outside influences play their role, but they probably do so by changing the mood of the individual bone cell and make him do this or that.

The bone cell s structural home consists of brick and mortar and one of his jobs in addition to providing rigidity to our frames is to give up and take up these bricks to maintain calcium homeostasis

It is hard to believe that this building and destruction of his home is not done from the inside more like a ship s interior that is he doesn t build or demolish his ship while bathing in sea water In other words his operations are performed behind a barrier influenced by factors in the sea or extracellular fluid but carried out for the most part by messages coming in by radio

That is the answer or the suggestion at least to the membrane theory it seems to me This is surely the way other tissues operate in any event

Dr Gersh made an analogy between bone and another fibrous tissue muscle and certain further analogies occur to one as follows By Dr Nordin s definition of porosis a total reduction of mass but normal construction of that remaining mass really means atrophy A muscle mass can be reduced by reducing arterial flow to it. Fibers are smaller but structurally the same

In the total individual we meet atrophy with seemingly normal structure in the queer psychologic disorder anorexia nervosa Here much less is

eaten, but a little of everything The person shrinks to 60 or 70 lb but is active and reasonably strong

By contrast, persons who eat far more calories than does the anorexia nervosa patient but lack one or more individual components of the normal diet suffer quite differently—for example those who starved in prison camps in the Second World War These persons suffered edema, low serum protein, and all sorts of dysfunctions which the anorexia nervosa patient does not show

Might we now make a further analogy with bone to the above? If subjected to reduction of arterial blood, he will atrophy as has been shown, just as will his muscle cousin. What would happen if he were deprived of individual items in his local dietary? This might be accomplished not necessarily by deficiency of the element in question in the circulating fluid, that is by a reduction in chemical pressure of such element, but might occur through some local problem at his very cell border

Indeed, one suspects that cortisone may act in such a way and I believe Dr Harrison sort of implied the same thing

Anything preventing access of a necessary constituent should alter the *cell behavior sharply An over all absence of a single constituent, such as* an essential amino acid, will produce atrophy of the entire body So too, Cannon found, will a deficiency of smaller chemical building blocks—potassium and phosphorus.

Now this is not meant as a theoretical substantiation of Dr Nordin s hypothesis that calcium lack is the cause of osteoporosis for I frankly don t believe it, myself The bone cell s interest in calcium is not for adequacy of his normal function He can carry out his routine duties unless serum calcium is extremely high He is interested in calcium as material for his house s wall

In the next few years one looks forward to clarification of some of the problems in our understanding of bone as a tissue which have been so variously presented at this conference After all that is what such a conference is for

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